ESTIMATING THE PREVALENCE OF VITAMIN B12 DEFICIENCY IN VEGETARIAN OUT PATIENTS BETWEEN 18 AND 60 YEARS OF AGE PRESENTING TO A TERTIARY CARE HOSPITAL

A Dissertation submitted in partial fulfillment of

M.D (General Medicine) branch I Examination of the Tamil Nadu

Dr. M.G.R. UNIVERSITY, CHENNAI

to be held in 2011.

C E R T I F I C A T E

This is to certify that the dissertation entitled "Estimating the prevalence of vitamin B12 deficiency in vegetarian out patients between 18 and 60 years of age presenting to a tertiary care hospital" is the bonafide original work of Dr. Anandaroop Lahiri, towards the M.D. Branch- I (General Medicine) Degree Examination of the Tamil Nadu Dr. M.G.R University, Chennai to be conducted in 2011.

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CONTENTS

AIMS

The primary aim of this study was to determine the prevalence of vitamin B12 deficiency among vegetarian out patients, between the age groups of 18 and 60 years, visiting the internal medicine out patient department of a tertiary level teaching hospital in South India, and to study their clinical profile along with the risk factors and clinical associations of this condition.

OBJECTIVES

The study was designed to accomplish the following six objectives:

A] To determine the prevalence of vitamin B12 deficiency among vegetarian patients (between the ages of eighteen and sixty years) coming to the internal medicine out patient department of Christian Medical College and Hospital, Vellore.

B] To study the clinical manifestations of vitamin B12 deficiency in vegetarians.

C] To study the risk factors associated with the development of vitamin B12 deficiency among vegetarians.

D] To study the awareness of vegetarians about vitamin B12 deficiency and the need for exogenous supplementation.

E] To study the different neuropsychiatric manifestations and the anxiety and the depression levels of the vitamin B12 deficient population

F] To make a preliminary observation as to the prevalence of surrogate markers for pernicious anaemia in the study population.

INTRODUCTION

Vitamin B12 is an essential nutrient that is used by the body to act as cofactors in certain key reactions within the cells. Cobalamin along with folate is needed for synthesis of DNA required in cells undergoing rapid turnover, such as haematopoetic and enteric lining cells. The physiological consequences of a deficiency in any of the above nutrients are increased homocysteine, reduced methionine and impaired formation of tetrahydrofolate. These changes ultimately lead to the characteristic neurological and haematological manifestations, not to mention certain events like vascular thromboses that may be seen even in the absence of overt vitamin B12 deficiency.

Animal products provide the only dietary source of vitamin B12. Vegetarianism is a well known risk factor for vitamin B12 deficiency. Other important etiological considerations are pernicious anaemia and malabsorption.

The treatment is simply supplementing the vitamin in the body with extremely satisfying results at minimum costs and adverse effects.

LITERATURE REVIEW

The concept of deficiency in vitamin B12 levels has been around in the Indian medical field for quite a long while. Renewed interest has however recently crept up again in this subject with the notion that this medical problem maybe more widespread and complicated than previously anticipated. Our knowledge about this subject is far from complete.

Historical perspective

The origin of the literature on vitamin B12 probably began with the earliest observations and descriptions of key manifestations of its deficiency in the nature of pernicious anaemia and subacute combined degeneration of the spinal cord.

The earliest published report of an illness akin to the complete spectrum of vitamin B12 deficiency has been documented by James Combe of Edinburgh (1) – however there was no confirmatory evidence of either pernicious anaemia or subacute combined degeneration in his report.

Subsequently in 1849 Thomas Addison first described pernicious anaemia (2) as a distinct entity. This was initially named Addisonian anaemia by Trousseau. Later Addison and Fenwick described the coexistence of anaemia, debility and gastric atrophy as a clinical syndrome (3) which unlike the other known anaemias at that point of time was almost always fatal.

Down the line scholars like Biermer, Leichtenstein, Gowers, Lichtheim, Hunter and Russell all made significant contributions to provide parts of the entire clinical

spectrum (4). Paul Ehrlich first in 1880 coined the term “megaloblast” (4) to describe the large cells in the bone marrow observed in the spectrum of vitamin B12 deficiency.

Nevertheless, the first person to convincingly propose a link between subacute combined degeneration and pernicious anaemia was probably James J Putnam (5). In 1890, he reported 8 “enfeebled patients” (7 ladies and 1 gentleman), with combined changes of the pyramidal tracts and the posterior columns along with evidence of neuropathy – these people in addition, also had anaemia and suffered from exhaustion. For a while this clinical entity it was known by the phrase “Putnam Dana syndrome”.

Interest in this field has been brewing in Christian Medical College, Vellore for around three decades now where the likes of Dr. Mathan and Dr. Baker have published extensively on the subject. Needless to say that information on this subject has come a long way since then.

Vegetarianism in India

Vegetarianism has been well known and commonly found in India since ancient times. It has probably in many ways got entangled with the socio-religious psyche of the people. A survey published in The Hindu (6) a few years ago claimed that 31% of Indians are pure vegetarians and another 9% are vegetarians who eat egg – in other words, 40% of the country does not consume meat or fish. The tendency towards vegetarianism apparently seems to be more in land-locked states like Rajasthan, Haryana, Punjab, Uttar Pradesh etc and less in the coastal states like Kerala, Andhra Pradesh, Tamil Nadu, West Bengal etc. People have also claimed that India houses more vegetarians than all

vegetarians put together all over the world. In view of the above, India becomes a nation likely to harbor vitamin B12 deficiency in profusion among the masses.

Epidemiology

The epidemiology of vitamin B12 deficiency has been changing from its first days of description. Factors that have been contributing to that are the various definitions both clinical and biochemical that have been proposed to define this condition. The wide array of available methods to objectively look for the levels of this vitamin and the various different sub-clinical forms of deficiency of this vitamin make its epidemiology even more heterogeneous.

World data

A worldwide prevalence for a condition such as this is probably difficult to come by – however discrete reports in various articles have quoted prevalence in various regions of the world. Review of such data reveals that the numbers differ in various age groups (7).

In the United States of America, reports of the National Health and Nutrition Examination Surveys performed between the years 1999 and 2002 have shown that vitamin B12 deficiency (vitamin B12 level less than 200 pg/mL) affected less than 3% of people between 20 to 39 years of age, around 4% of those between 40 to 59 years of age and around 6% of those above the age of 60 years (8; 9). Borderline levels (vitamin B12 between 200 and 300 pg/mL) were commoner in the same population – about 14-16%

amongst those aged 20to 59 years of age and more than 20% amongst those above 60 years of age.

In the United Kingdom, 1 in 20 people in the age group of 65 to 74 years of age and about 1 in 10 people above the age of 75 years had this condition (10; 11).

In Mexico according to the Mexican National Nutrition Survey, performed in 1999 the prevalence of deficiency and marginal levels, was around 40% of all children and adults (12).

Talking about Africa, 70% of Kenyan children were found to be deficient (13).

In 2003, Herrmann et al (14) studied 174 apparently healthy subjects (66 people who were vegetarians with milk products and egg consumption, 29 vegans, and 79 people who were non vegetarians) living in Germany and Netherlands. 52% of the vegans had absolute deficiency of vitamin B12 and around 90% had indirect metabolic features suggestive of vitamin B12 deficiency (like elevated methylmalonic acid and homocysteine). 26% of the people who were vegetarians with milk products and egg consumption were deficient in vitamin B12 and only 1% of the non vegetarians were deficient in vitamin B12.

Indian data

Community level data is not very forthcoming in the literature. However there is some data from India about community prevalence. In Pune, Maharashtra, India, Yajnik et al performed a study (15), where they randomly selected people within the age groups of 30 and 50 years from 2 villages, 2 slums and 2 middle class regions around Pune. They studied 441 subjects in all and the overall pooled prevalence of vitamin B12 deficiency

was 67%. Vegetarians had a three fold higher risk of having vitamin B12 deficiency as non vegetarians.

A study by Jathar et al (16) in 1975 amongst healthy medical students had shown vitamin B12 deficiency to be prevalent in 47% of people taking a pure vegetarian diet along with milk products.

In 1985 Chanarin et al published a study (17) where they studied 138 Indian patients with megaloblastic haemopoiesis. Among them there were 95 people (68%) who were deficient in vitamin B12. All the 138 patients were vegetarians. 20 of the vitamin B12 deficient people had pernicious anaemia.

In 2001, Refsum et al (18) studied 204 men and women in Maharashtra, India for vitamin B12 deficiency. Of them 47% were found to have deficient vitamin B12. What is interesting is that in the same study the investigators went on to study the indirect metabolic indicators of vitamin B12 deficiency like raised homocysteine levels and raised methylmalonic acid levels and found "Functional vitamin B12 deficiency" in 77% of the subjects. This study tells us two things – firstly in any study where we are assessing the prevalence of vitamin B12 deficiency with absolute vitamin B12 levels, there is bound to be underestimation of its prevalence, and secondly the clinical spectrum of vitamin B12 deficiency possibly begins long before the actual fall in the levels to the point of absolute deficiency.

Previously unpublished data from our centre that had looked at an elderly population of patients (200 patients with age greater than 60 years) with frank dementia found that 38 patients (19%) had vitamin B12 deficiency.

In a pilot study, done as a prelude to this study, in our own hospital, among fifty new vegetarian outpatients, 22 (44%) were found to be deficient in vitamin B12 levels. While doing this analysis there were quite a few people who were non vegetarians but were still found to have low vitamin B12 levels – hence there is probably a sizeable population amongst non vegetarians as well that will probably be deficient in this vitamin that requires investigating at a later stage.

Apart from this there is a great dearth of any further data on the prevalence (be it community based or hospital based), of vitamin B12 deficiency in the country. The quoted percentages seem to be distinctly higher than any other such value from anywhere else in the world. Is it representative of the entire country? We would probably need to anticipate for more data in this area before any definitive conclusions can be drawn regarding this issue.

Etiology of vitamin B12 deficiency

Traditional causes of vitamin B12 deficiency are well known and well studied. However newer factors attributing to this deficiency are being proposed by the day. The following table (refer table 1) is an attempt to summarize the well addressed causes under one heading.

Table 1: Etiology of vitamin B12 deficiency

Pernicious anaemia
Autoantibody formation
Chronic atrophic gastritis
Helicobacter pylori
Cobalamine malabsorption
Gastric atrophy, achlorhydria
Helicobacter pylori infection
Intestinal bacterial overgrowth secondary to antibiotic treatment
Long-term ingestion of biguanides, antacids, H2 receptor antagonists, and proton pump inhibitors
Chronic alcoholism
Gastric surgery/reconstruction for obesity (bariatric surgery)
Pancreatic exocrine failure
Sjogren's syndrome
Ileal disease (including tuberculous ileitis, lymphoma, amyloid, long-term survivors of pelvic irradiation), resection or bypass, and Crohn's disease
Zollinger Ellison syndrome
Gluten induced enteropathy
Infections
Diphyllobothrium latum
Human immunodeficiency virus
Hereditary conditions
Qualitative abnormalities of intrinsic factor
Imerslund-Grasbeck's disease or juvenile megaloblastic anaemia
Congenital deficiency of transcobalamin
Homocystinuria
Severe methylenetetrahydrofolate reductase deficiency
Abnormalities of methionine synthesis
Abnormal lysosomal membrane exporter for cobalamin
Nitrous oxide exposure
Radiotherapy
Graft versus host disease
Poor dietary intake

Risk factors for vitamin B12 deficiency amongst vegetarians

The reason for low levels in vitamin B12 deficient vegetarians is most likely due to an absence of vitamin B12 in a pure vegetarian diet. But is that the whole truth? Could there be other associated or superadded factors that contribute to it? The fact that not all

vegetarians are deficient, added to the fact that even non vegetarians suffer from severe deficiency, lets one to the belief that there must be other factors involved in this process rather than diet solely.

Water

There is a study by Mathan et al (19), done long back, which demonstrated that small quantities of vitamin B12 are present in drinking water probably due to faecal contamination of the water. Hence the possibility remains that a part of the population derives its share of vitamin B12 actually from the water itself. If we consider that, in the entire community there has been a general trend towards shifting towards more and more purified sources of drinking water, then it is possible to consider that people using more purified sources of water, are at a higher risk of acquiring this deficiency compared to people who use less pure forms of drinking water. This would be a hypothesis by itself as there is not much literature to go by in this field apart from Dr. Mathan's study (19). This is one factor that we looked at in our current study to check if the nature of the drinking water had any bearing on the prevalence of vitamin B12 deficiency.

Pernicious anaemia

Pernicious anaemia is known to be a cause of vitamin B12 deficiency (20). The possibility remains, in this situation, of Occam's razor being blunted and coexistence of pernicious anaemia along with vegetarianism leading to vitamin B12 deficiency. Is there any coexistence of the two in an Indian population – no one really knows. In the study by Jathar et al (16), Schilling tests were carried out on his group of medical students and

there was enough evidence to say at least that the intestinal absorption of vitamin B12 is similar amongst the vegetarians and the non vegetarians. However as to the exact question of whether there is coexisting pernicious anaemia, there is no answer. In an attempt to address this point we tried to look for any evidence of the possibility of the co-occurrence of pernicious anaemia in this group of people.

The diagnosis of pernicious anaemia is made by a combination of macrocytic anaemia, documented vitamin B12 deficiency, demonstration of atrophic body gastritis, and intrinsic factor deficiency (20). However indirect evidence of such a disorder may be obtained with the help of intrinsic factor antibodies and parietal cell antibodies. How good would they be in predicting this condition? This issue has been assessed recently and it has been found that intrinsic factor antibodies have a sensitivity and a specificity of 37% and 100% respectively while anti parietal cell antibodies have a sensitivity and specificity of 81.5% and 93.3% respectively (21). In our study we used these antibodies as possible surrogate markers for pernicious anaemia. Our study was limited by the lack of funds to do the entire evaluation required for the patients in order to prove a diagnosis of pernicious anaemia conclusively.

Drugs

Usage of certain drugs has been in the past studied in association with vitamin B12 deficiency. In one study, 53 vitamin B12 deficient patients were compared with 212 controls, all above the age group of 65 years for past or current use of either of H2 receptor antagonists or proton pump inhibitors according to review of the patient's medical records (22). Once factors like age, gender, multivitamin use and Helicobacter

pylori infection were controlled for, chronic or current use of the above drugs were significantly associated with an increased risk of vitamin B12 deficiency (odds ratio 1.47-13.34). In another report (23) that looked at a cross-section of elderly people with respect to the use of acid suppression and vitamin B12 deficiency, it was found that proton pump inhibitors but no H2 receptor blockers were statistically associated with the risk of vitamin B12 deficiency. We also looked at whether significant acid suppression existed within our study population and whether that in any way related to vitamin B12 deficiency.

There is data now at the level of a randomized trial to suggest that metformin use is a risk factor for developing vitamin B12 deficiency. In this multi centre randomized placebo controlled trial (24), 390 patients with type 2 diabetes mellitus on insulin, were randomized into metformin (850mg three times a day) and placebo. The main outcome measure was the percentage change in vitamin B12 levels at various intervals till 52 months of follow up. Compared with placebo, treatment with metformin was associated with a statistically significant mean decrease in vitamin B12 levels of 19%. This trial was published in 2010 and actually when we started our study this trial had not been published. Hence we also looked at whether metformin was significantly associated with vitamin B12 deficiency.

Others

The other possibility is to consider whether there could be some inhibitors in a vegetarian diet that would lead to inadequate absorption of the vitamin. Now this is absolutely new ground here where there is no data at all whatsoever about this possibility.

Indians are known to chew pan and betel leaves and the like and these practices are common in the vegetarian population. Hence we conjured that it may be worth studying if pan, betel leaves and other such agents have any association with vitamin B12 deficiency.

Absorption and handling of vitamin B12

Dietary cobalamin after ingestion reaches the stomach and in the presence of acid and pepsin in the stomach is liberated from binding to protein and then quickly binds to R factors (cobalamin-binding proteins) in saliva and gastric juice. Cobalamin bound to R factors is not absorbed; however, in the alkaline pancreatic enzyme environment of the duodenum, cobalamin is freed from R proteins by pancreatic proteases and then binds specifically and rapidly to gastric-derived intrinsic factor. Intrinsic factor is a 45 kilo Dalton glycoprotein with very high affinity for cobalamin.

The intrinsic factor - cobalamin complex binds to a specific ileal receptor, cubilin, from which it is absorbed via an energy requiring process that is still not completely understood (25-27). Electron microscopic studies have shown colocalization of cubilin with the endocytic proteins megalin and "amnionless" (AMN), which may mediate vesicular trafficking of the complex, via a calcium-dependent mechanism (28). It is possible that the functional ileal receptor for the cobalamin-intrinsic factor complex is a complex of AMN and cubulin (29).

Mutations in either the cubilin gene or the AMN gene can cause hereditary megaloblastic anemia, while absence of megalin has been associated with failure of

normal renal tubular reabsorption of the Cobalamin-transcobalamin complex in mice (30).

Thus, adequate absorption of cobalamin depends upon five factors:

* Adequate dietary intake

* Acid-pepsin in the stomach

* Pancreatic proteases

* Gastric secretion of a functional intrinsic factor

* An ileum with functioning cobalamin-intrinsic factor receptors

The need for an intact upper gastrointestinal tract for effective absorption of cobalamin and folic acid has been shown in a report of patients receiving a Roux-en-Y gastric bypass for morbid obesity. Standard multivitamin preparations were inadequate to maintain vitamin B12, folic acid, iron, calcium, and vitamin D levels (31).

After being taken up by ileal enterocytes, cobalamin is exported via the ATP-binding cassette (ABC)-drug transporter ABCC1 (also called multidrug resistance protein, MRP1), present in the basolateral membrane of intestinal epithelium and other cells (32). Cobalamin enters plasma, bound to three transcobalamins: transcobalamin I, II, and III. Up to 80 percent of cobalamin is bound to transcobalamin I and III, which have no identified role in cobalamin metabolism (33). It is the transcobalamin II-cobalamin complex that is physiologically important. The three-dimensional structure of transcobalamin shows that there are 2 domains for binding cobalamin (29). This complex has a half-life of six to nine minutes and binds to specific cell surface receptors from which it enters cells by receptor mediated endocytosis. Cobalamin in the cells is

metabolized into two coenzymes: adenosyl-cobalamin; and methyl-cobalamin, the functions of which are described below (33).

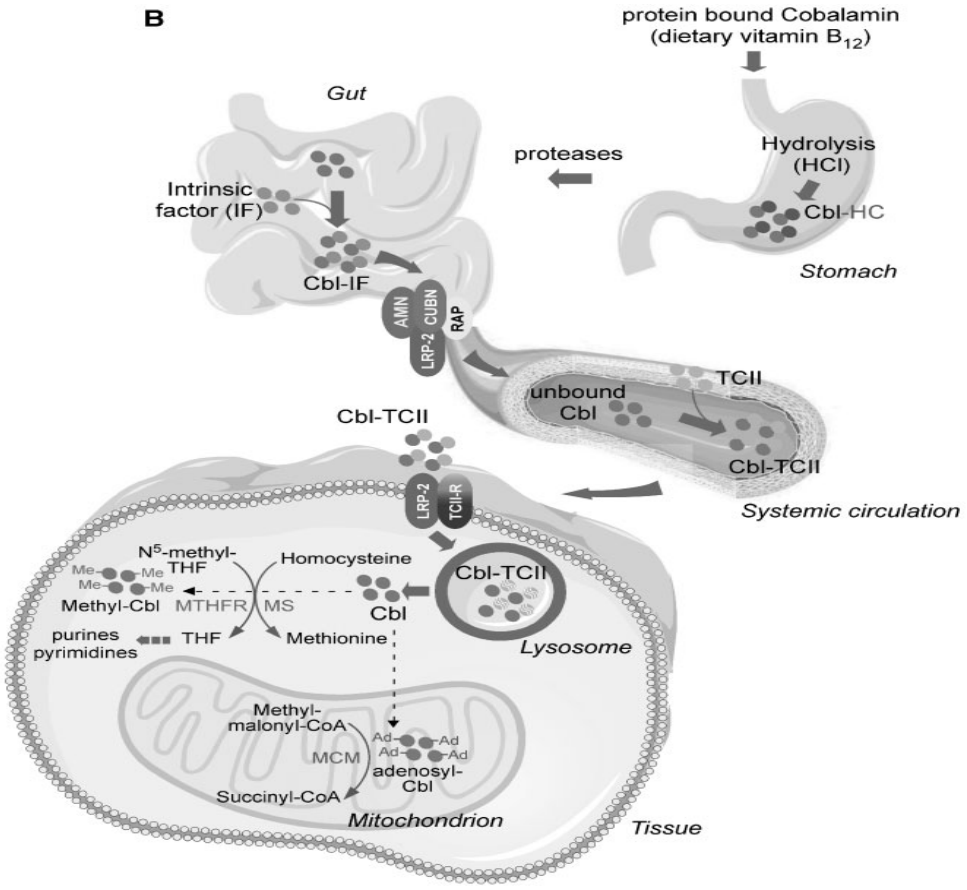

Figure 1: Handling of vitamin B12 (34)

Physiological role of vitamin B12

The concerted action of these two vitamins leads to the DNA synthesis required in cells undergoing rapid turnover, such as hematopoietic and enteric lining cells. Although the exact steps remain elusive, cobalamin has two known cofactor actions as shown below (refer figure 2).

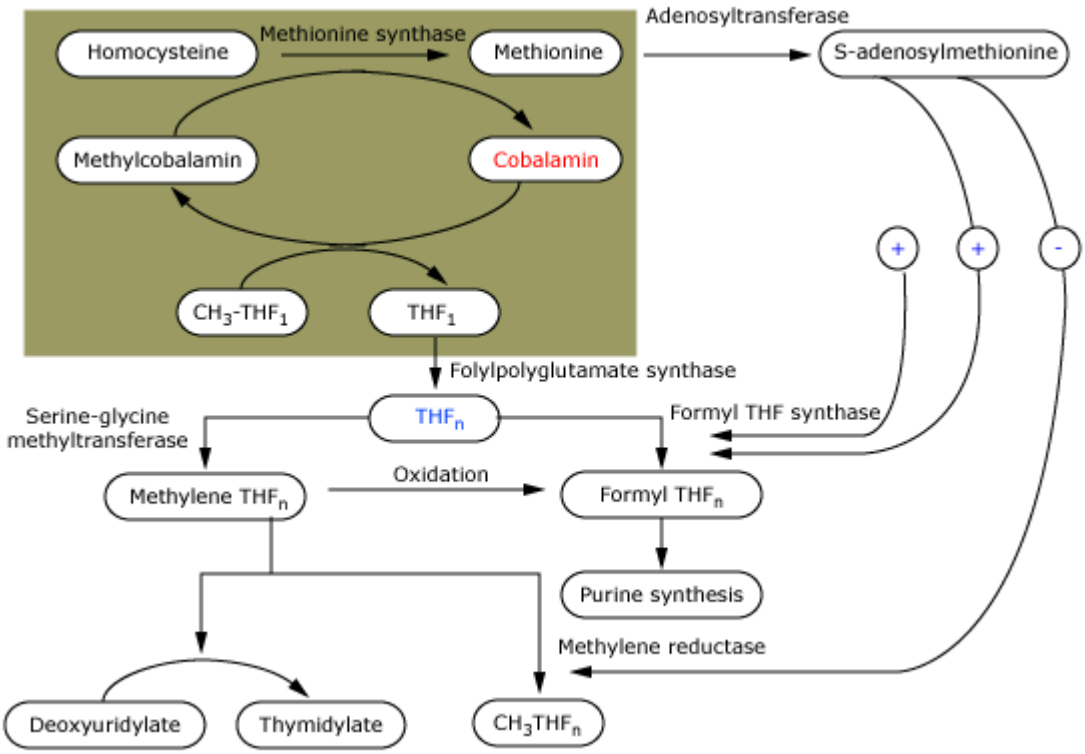

Figure 2: Physiological role of cobalamin and folate (33)

Conversion of propionyl-CoA to methylmalonyl CoA and finally to succinyl-CoA — the biologic significance of this sequence remains unknown. There is no interaction with folic acid in this pathway; as a result, it has been proposed that this pathway might be important in myelin formation and in the neurological abnormalities typical with vitamin B12 but not folic acid deficiency (33). However, the observation that hereditary deficiencies of the enzyme methylmalonyl CoA mutase do not cause neuropathy is not consistent with this hypothesis.

Transfer of a methyl group from methyl-tetrahydrofolate (methyl-THF) via cobalamin to homocysteine to form methionine — this reaction has two important effects: it reduces the plasma concentration of homocysteine, which is probably toxic to endothelial cells; and, perhaps more importantly, it demethylates THF. Demethylation is

a critical step in DNA synthesis because THF (the reduced form of folate) and not methyl-THF is the substrate for the enzyme that converts (THF)-1 to the polyglutamated form, (THF)n.

Only polyglutamated (THF)n participates in purine synthesis and in converting deoxyuridylate to thymidylate via the transfer of 1-carbon units (33). As an example, methylene-(THF)n transfers a methylene molecule to convert deoxyuridylate to thymidylate. Methylene (THF)n can also be oxidized to formyl (THF)n, which is important in purine synthesis.

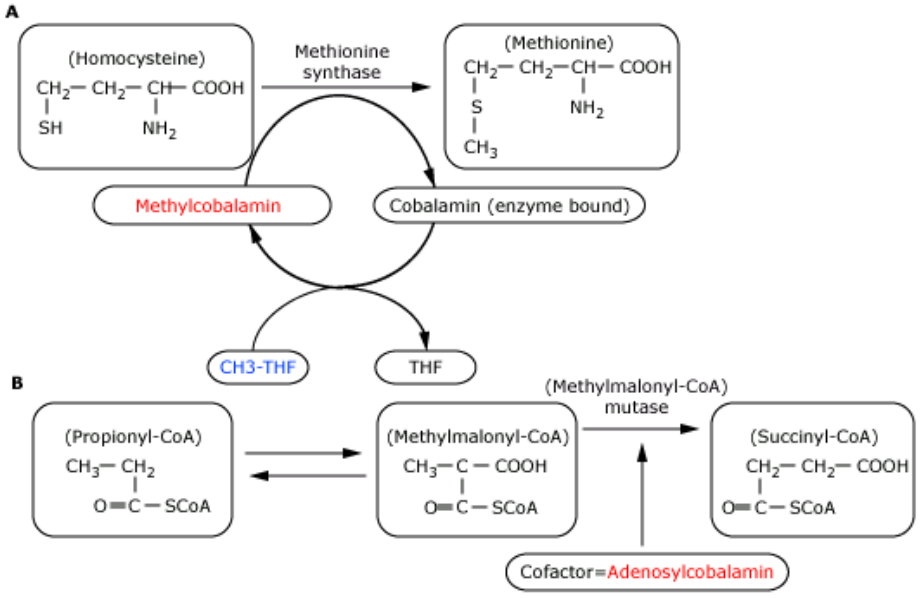

Figure 3: Role of cobalamin (33)

Effects of cobalamin and folic acid deficiency

There are three main consequences:

* Increased homocysteine levels

* Reduced methionine levels

* Impaired formation of THF

Two observations suggest that methionine deficiency may play a major role in the neuropathy associated with cobalamin deficiency. First, neuropathy can be produced by toxic exposure to the gas nitric oxide (NO), which inhibits methionine synthase. Second, methionine administration has been beneficial in an animal model of cobalamin neuropathy (35). The mechanism by which methionine deficiency causes neuropathy and why neuropathy does not occur with folate deficiency remain unclear.

In addition, adult cobalamin-deficient patients have high levels of TNF-alpha and low levels of EGF in the serum and cerebrospinal fluid; these levels normalize along with hematologic disease remission following treatment. Hence these molecules might also be involved in the pathophysiology of this process.

Pathophysiology of megaloblastosis

Deficiency of cobalamin and folic acid leads to megaloblastic erythropoiesis and frequently to disordered maturation in the granulocytic and megakaryocytic lineages. The ultimate basis for the megaloblast is inadequate conversion of deoxyuridylate to thymidylate, which leads to slowing of DNA synthesis and delayed nuclear maturation. RNA and protein synthesis proceed normally, resulting in the characteristic "cytonuclear" dissociation of the megaloblast.

There are two hypotheses concerning the exact mechanism by which these vitamin deficiencies slow DNA synthesis (figure 3):

* In cobalamin deficiency, methyl-THF cannot be demethylated; as a result, there is no THF available for the critical polyglutamation step discussed above. This is the "methylfolate trap" hypothesis, which can theoretically explain many of the metabolic

abnormalities identified (33). However, demethylated-THF does not correct these abnormalities, raising a question about the validity of this hypothesis (33).

* An alternative explanation, the "formate starvation hypothesis", proposes that methionine deficiency is the primary problem. Support for this hypothesis comes from the partial correction of many of the metabolic abnormalities with methionine and from the observation that methionine functions to enhance generation of formyl-(THF)n either directly or when converted to S-adenosyl methionine (figure 3) (33).

Ineffective erythropoiesis

Regardless of the mechanism, the morphologic hallmark of cobalamin and folate deficiency is megaloblastic erythropoiesis, which probably reflects the defective DNA synthesis. The kinetic and morphologic cause of the anaemia is ineffective erythropoiesis or intramedullary hemolysis. This means that there is intense erythroid hyperplasia in the marrow but relative reticulocytopenia. Thus, the erythroid precursors are not maturing normally and are dying in the bone marrow, resulting in the lack of orderly delivery of red cells into the peripheral blood.

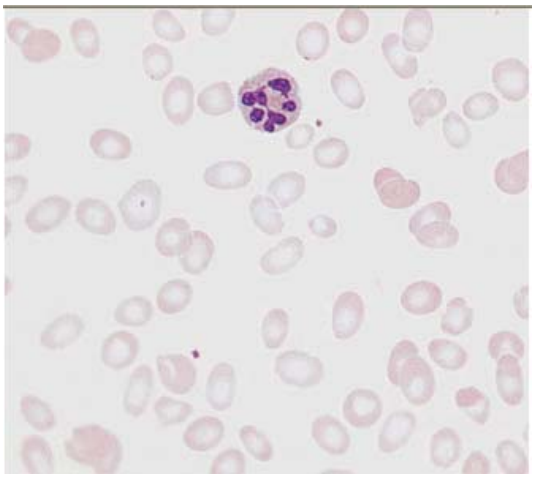

Figure 4: Hypersegmented neutrophil

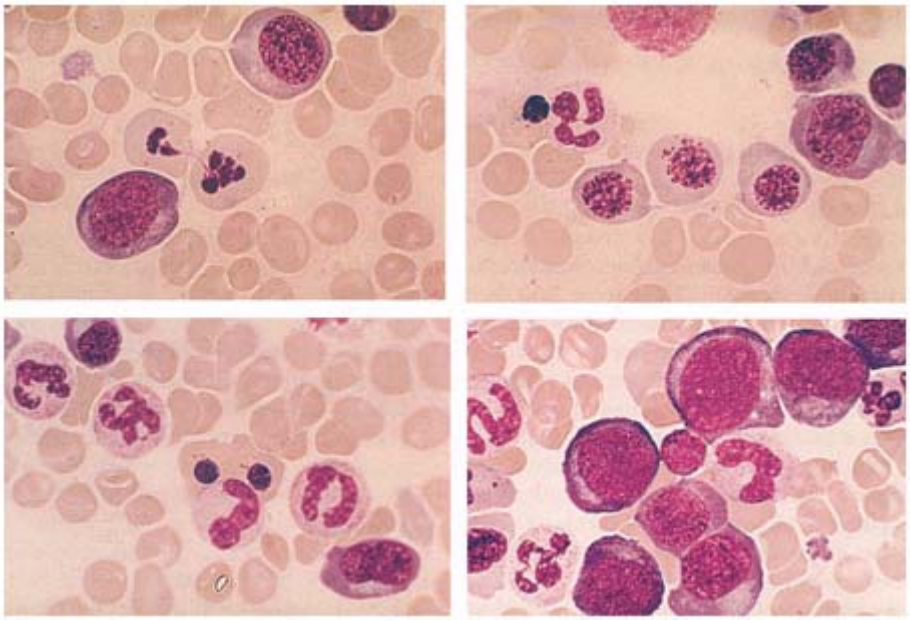

Figure 5: Megaloblastic eryhthropoesis

The underlying mechanism of ineffective erythropoiesis, as tested in an animal model of folate deficiency, is enhanced apoptosis (36). The apoptosis is prevented in this model by thymidine administration, thereby emphasizing the importance of the block in the synthesis of thymidylate (36). However, in patients with megaloblastic erythropoiesis due to cobalamin or folate deficiency, intramedullary death of RBC precursors is not characterized by apoptosis (37; 38), and requires a different explanation.

Ineffective megakaryocytopoiesis — The same defect presumably occurs in myeloid and megakaryocytic precursors. In severe cobalamin deficiency, the low platelet count is associated with a marked increase in the number of megakaryocytes but diminished ploidy, resulting in an expanded megakaryocyte mass but reduced platelet production per megakaryocyte.

Manifestations of vitamin B12 deficiency

Physical characteristics of vitamin B12 deficiency could be markedly varied. Sometimes the features could be remarkably subtle (34) – so subtle in fact as to merit being labeled asymptomatic. They two most prominent and potentially serious presentations are haematological and neuropsychiatric. Newer hypotheses about end organ damage are being considered in the literature every day.

Mucocutaneous manifestations

Mucocutaneous changes may be picked up on careful examination in people with vitamin B12 deficiency. Some of them may not be very specific. But they may be clues to the possible underlying diagnosis of cobalamin deficiency.

The pigmentation of vitamin B12 deficiency is generally markedly pronounced in the hands and the feet and especially in the creases of the palmar and the plantar aspects. Sometimes hyperpigmentation has been seen to be accentuated over the terminal phalanges and over the pressure points such as the elbows, malleoli and the knees. Rarely excess pigmentation has also been noticed in the buccal mucosal membrane with spotty pigmentation of the tongue (39). Nails can show longitudinal hyperpigmented streaks, but the nail beds may actually be pale. One paradoxical finding in terms of pigmentation maybe early graying; this has also been described (39).

The mechanism of hyperpigmentation may be most likely associated with alterations with tyrosine levels (40). A deficiency of vitamin B12 causes a decrease in reduced glutathione levels, and tyrosinase, an enzyme necessary for melanogenesis, is inhibited by reduced glutathione. Therefore the decrease in reduced glutathione levels in

vitamin B12 deficiency leads to an increase in tyrosinase levels, giving rise to hypermelanosis.

Changes in the oral mucosa in vitamin B12 deficiency maybe important as many of these features predate the systemic manifestations of this condition (41). A wide range of oral signs and symptoms may appear in such patients as a result of basic changes in the metabolism of oral epithelial cells. These changes give rise to abnormalities in cell structure and the keratinization pattern of the oral epithelium leading to a “beefy” red and inflamed tongue with erythematous macular lesions on the dorsal and border surfaces because of marked epithelial atrophy and reduced thickness of the epithelial layer (42).

Haematological manifestations

Macrocytic anaemia

Macrocytosis has been defined as a mean corpuscular volume greater than 100fl (43). Apparently it can be found in about 3% of the normal population (43). Macrocytosis can be due to a host of causes, B12 deficiency being just one amongst them.

Causes of macrocytosis: (43)

Table 2: Etiology of macrocytosis

Megaloblastic (involving vitamin B_{12} and/or folate deficiencies)
Atrophic gastritis
Enteral malabsorption
Human immunodeficiency virus treatments
Anticonvulsants (some cause folate depletion)
Primary bone marrow disorders
Nitrous oxide abuse
Inherited disorders
Nonmegaloblastic
Alcohol abuse
Medication side effects (see Table 3)
Myelodysplasia
Hypothyroidism
Liver disease
Hemolysis
Hemorrhage
Chronic obstructive pulmonary disease
Splenectomy
False elevations
Cold agglutinins
Hyperglycemia
Marked leukocytosis

Evaluation of macrocytosis can be done based on the following algorithm: (43)

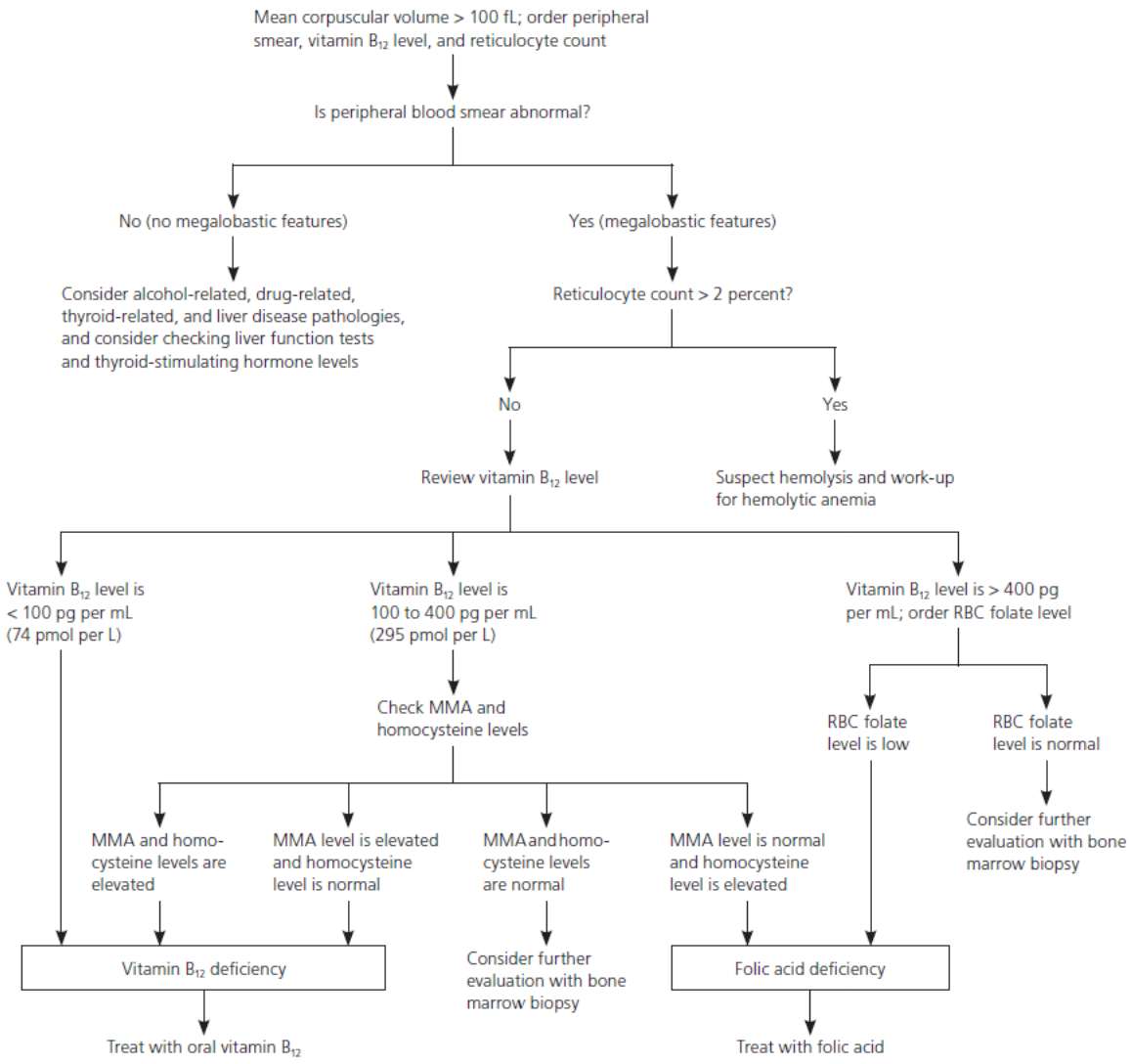

Figure 6: Algorithm for evaluation of macrocytosis

Vitamin B12 deficiency is a well known cause of macrocytic anaemia with evidence of haemolysis. It is possible for it to present even as frank pancytopenia (44). Strangely enough the haematological abnormalities may not be seen in all patients with deficient vitamin B12 levels. In Dr. Yajnik's study, though 67% of the subjects had low vitamin B12 concentration, only 2% had an elevated mean corpuscular volume (15). This could be due to the fact that there may have been coexisting iron deficiency also in the population. Andres et al (45) studied the haematological manifestations of 201 vitamin

B12 deficient people and found that anaemia was present in 37% of the subjects and macrocytosis in 54%. In the pilot study done in our hospital only 4 of the fifty patients looked at, had a mean corpuscular volume of 90 and above (8%), and 13 of the fifty (26%) had anaemia. Hence from the above reports it looks as though vitamin B12 deficiency can present in subtle ways with no change in the mean corpuscular volume or the blood picture. This basically goes on to indicate that the profiles of these haematological manifestations are not very clearly defined – nor do they seem to be uniform. Hence the threshold for suspecting vitamin B12 deficiency may be actually quite low and probably a normal mean corpuscular volume in the blood work might not rule out vitamin B12 deficiency. We therefore, looked at the possibility of whether there could be a value for the mean corpuscular volume that could actually predict vitamin B12 deficiency with a reasonable degree of certainty in our study population.

Vascular thrombosis

We know that vitamin B12 deficiency is accompanied by hyperhomocysteinemia and elevated methyl malonic acid levels in the serum. The serum levels of homocysteine and methyl malonic acid actually rise earlier than the actual dip of vitamin B12 values to absolute deficient levels – some people have termed this as “Functional vitamin B12 deficiency” (18). Hyperhomocysteinemia has been implicated as a risk factor for thrombotic disease (46). There are a handful scattered reports in the literature where vitamin B12 was evidently causatively associated with thrombosis (47). This raises the issue of whether regular screening for vitamin B12 levels would be necessary for evaluating any patient with an unprovoked thrombosis with no other vascular risk factors.

In this context it would be interesting to study whether such manifestations are evident in our population.

Other haematological manifestations

Apart from macrocytosis, other unusual haematological manifestations that have been described in the literature are severe pancytopenia, splenomegaly, mild icterus and leucoerythroblastosis (48). There can be circulating immature sells as well on the peripheral blood smear (48). These findings having been described in the past – however in the recent past they have not really been prominently portrayed. These maybe markers of severe megaloblastic erythropoesis and must be kept in mind when looking for haematological abnormalities associated with this condition.

Neuropsychiatric manifestations

Neuropsychiatric and neurological manifestations of vitamin B12 deficiency are well known but are varied and with time newer features and associations are being described (49; 50).

Neurological syndromes

Amongst the common neurological manifestations is peripheral neuropathy that has been well documented (51). Dorsal myelopathy in the form of the typical sub acute combined degeneration is also well studied (52). In subacute combined degeneration there is generally an involvement of both the posterior columns (dorsal columns) as well as the lateral cords (pyramidal system). These lesions specific for vitamin B12 deficiency

are due to a defect in myelin formation of unknown mechanisms. The neuropathy is symmetrical and affects the legs more than the arms. It begins with paraesthesias and ataxia associated with loss of vibration and position sense, and can progress to severe weakness, spasticity, clonus, paraplegia, and even fecal and urinary incontinence (53). Optic neuropathy though very rare also has been described in cobalamin deficiency as a cause of painless progressive loss of vision (54). Most of the neurological manifestations if picked up fairly early in the disease can be reversible with adequate and appropriate supplementation.

Dementia

There are only a few reversible causes of dementia in the elderly of which Vitamin B12 deficiency is the most easily treatable one. Vitamin B12 deficiency can cause isolated dementia or can be a coexisting factor in other irreversible dementias.

The first major study which looked into various causes of dementia in elderly (>60yrs) in India was from SGPGI hospital (55). It was a prospective study conducted on 124 (94 male and 30 female) elderly patients (aged more than 60 years) presenting with clinical syndrome of dementia (MMSE less than 24). Their age range was 64-78 (mean 65.7 4.1) years. Multi-infarct dementia (MID) was observed to be commonest cause of dementia and was present in 59 (47.6%) cases followed by,10 (8%) patients each of tuberculosis (TB) and neurocysticercosis (NCC), Alcohol-related dementia in 13 (10.5%),malnutrition (Vitamin B12 deficiency) was present in 9 (7.2%), Alzheimer's disease (AD) in 6 patients (4.8%),1 each of Huntington's disease, Parkinson's and Normal Pressure Hydrocephalus and 2 each of diabetes, hypothyroidism, hyperthyroidism and

Creutzfeldt' Jakob Disease. It was concluded that AD, which is irreversible and common in the west, is relatively uncommon in India as compared to M.I.D, infections and malnutrition, which are potentially treatable. In its classic form dementia associated with B12 deficiency presents as subcortical dementia.

Psychiatric syndromes

In our experience in the out patient department of our hospital we had been seeing a number of patients with B12 deficiency presenting with multiple somatic complaints (bordering on anxiety/depression spectrum). So we wondered whether there were other as to date not well studied neuropsychiatric rather than pure neurological features of this condition.

Strangely enough a review of the literature revealed that psychiatric symptoms attributable to vitamin B12 deficiency had been described for decades. These symptoms seem to fall into several clinically separate categories: slow cerebration, confusion, memory changes, delirium with or without hallucinations and or delusions, depression, acute psychotic states, and more rarely, reversible manic and schizophreniform states (56). A higher prevalence of low serum vitamin B12 levels have been found in subjects with Alzheimer's disease and other dementias and in people with different cognitive impairments, as compared with controls (57). Furthermore, some interventional studies have shown the effectiveness of vitamin B12 supplementation in improving cognition in demented or cognitively impaired subjects. Martin D C et al reported a study in 1992, where twenty-two subjects with low serum vitamin B12 levels and evidence of cognitive dysfunction, were recruited consecutively over an 8-month period of time. Subjects

received 1000 micrograms of cyanocobalamin intramuscularly daily for 1 week, then weekly for 1 month, then monthly thereafter for a minimum of six months. Patients symptomatic for less than 12 months gained an average of twenty points on the Mattis Dementia Rating Scale (paired t test P = 0.0076), whereas patients symptomatic greater than 12 months lost an average of three points (paired t test P = .34) (58), indicating benefit with early replacement therapy.

Dementia is increasingly being picked up in the population. To quote an example, a three year epidemiological survey (59) was carried out in an urban community setting in Mumbai, India, where the prevalence of dementia was determined. The prevalence rate for dementia in those aged 40 years and more was 0.43%, and for persons aged 65 and above was 2.44%. The most common cause of dementia identified in this particular study was Alzheimer's disease followed by vascular dementia being the second most common cause.

In one series (60) where the investigators had studied 129 consecutive patients presenting to a hospital and fulfilling criteria for dementia, 24 patients had reversible causes of dementia of which only 5 patients had vitamin B12 deficiency. In previously unpublished data from our very own hospital, where 200 patients in the age group more than 60 years of age, with DSM IV criteria proven dementia were studied, it was found that around 10 % of the patients had dementia solely due to vitamin B12 deficiency and the overall incidence of vitamin B12 deficiency was around 19%.

Hence there is a need to actively look out for these uncommon neuropsychiatric and neurological manifestations for their further characterization. This is necessary in order to lower the threshold for the detection of vitamin B12 deficiency. Systematic data

regarding the prevalence of anxiety and depression amongst the b12 deficient is not really available in the literature.

Treatment of vitamin B12 deficiency

Parenteral cobalamin — Pernicious anemia is typically treated with parenteral (i.e., intramuscular or deep subcutaneous) cobalamin, in a dose of 1000 micrograms (1000 mcg, 1 mg) every day for one week, followed by 1 mg every week for four weeks and then, if the underlying disorder persists (e.g., pernicious anaemia, surgical removal of the terminal ileum), 1 mg every month for the remainder of the patient's life. If the cause of the cobalamin deficiency can be eliminated (e.g., diet, drugs, reversible malabsorption syndromes), treatment can be stopped when the cobalamin deficiency has been fully reversed and the cause eliminated.

While doses lower than those noted above have been recommended (i.e., 100 micrograms in place of 1,000 micrograms), there are few adverse consequences of this potential "overtreatment", as parenteral vitamin B12 is inexpensive, fairly nontoxic, and amounts given in excess of need are excreted harmlessly in the urine. Conversely, use of the lower dose could result in a slower response, which might be critically disadvantageous when severe neurological disease (e.g., subacute combined degeneration) is present and avoidance of irreversible neurological damage is a concern (52).

Oral and nasal formulations — An alternative that appears to be as effective as parenteral therapy, but which requires much greater patient compliance, is high dose oral cobalamin. The rationale for this approach in patients with impaired intrinsic factor

function is the presence of a second, lower efficiency transport system for cobalamin that does not require intrinsic factor or a functioning terminal ileum. This system consistently produces adequate long-term vitamin B12 replacement at doses of 1000 to 2000 mcg/day. Because of variability in absorption, lower oral doses are not completely effective in some patients with pernicious anemia (61).

The dose given in this situation (1 to 2 milligrams/day) is more than 200 times higher than the minimum daily requirement for normal subjects (62), and significantly higher than that available in most standard multivitamins and B12 supplements (≤100 mcg/day) (63).

In the few randomized clinical trials which have been reported, the use of oral cobalamin (1000 to 2000 mcg/day) in newly diagnosed patients was found to be as effective as intramuscular administration in obtaining short-term haematological and neurological responses in vitamin B12-deficient patients (64-66).

Because of the possibility of erratic absorption, it is most appropriate to use this route of treatment after the patient's cobalamin status has been normalized with parenteral treatment and/or to monitor the response frequently with determinations of serum cobalamin and methylmalonate concentrations.

Cobalamin can also be given sublingually (67), or via a nasal spray or gel (68). Sublingual and nasal routes of treatment have not been adequately studied and the available formulations are expensive (69).

Awareness regarding vitamin B12 deficiency

Often we see in the outpatient department that vegetarians are not aware of this condition of vitamin B12 deficiency. Vegetarians especially maybe a subset of the population who may benefit from education on this front. A lot of them may not actually be on supplementation. There is absolutely no data about the awareness of this condition in the Indian population. Sometimes there maybe a dearth of awareness of this even among various sections of the medical community. Unless it is shown consistently in Indian studies that this is a potential problem, the awareness may continue to be low. Hence an assessment of the level of knowledge amongst the subjects themselves maybe of the essence to take the first step towards making this an important public health problem.

So it would seem to suggest that though the problem of Vitamin B12 surfaced in the early 1970s there have been only very few studies substantiating the prevalence of the disease, making this a relevant point of further studies. Also the indirect evidence that can be possibly gathered from all the above mentioned studies would indicate that the prevalence of the problem may actually be quite high.

The clues to vitamin B12 deficiency in the out patient department though occasionally glaringly simple might in many situations be more subtle and easily missed - looking at the prevalence of anaemia in vitamin B12 deficient people. This further drives home the point that among the varied spectrum of manifestations of vitamin B12 deficiency there maybe no evidence of anaemia, with other features being present, even with very low levels of vitamin B12. This group would include a lot of fairly

asymptomatic individuals with low vitamin B12 levels – the exact clinical correlates of such a situation remain to be clearly elucidated.

In this study we will try to look at the prevalence of vitamin B12 deficiency among new vegetarian patients visiting the internal medicine out patient department of the Christian Medical College and Hospital, Vellore. We will also look at the clinical profile of the patients and try to identify possible uncommon neuropsychiatric manifestations of this condition like anxiety and depression. We will also study if there are any significant differences between deficient subjects and normal subjects with respect to exposure factors like the nature of the drinking water, the use of betel leaves and jarda, the use of proton pump inhibitors / H2 receptor blockers, and the use of aspirin or metformin. We will also try to look at the awareness of the vegetarians regarding this condition.

MATERIALS AND METHODS

Study design

This study was carried out based on the principles of a cross sectional study design. It involved observation and obtaining scientific readings at a particular point in time, with no follow up, either prospectively or retrospectively. A cross sectional study is amenable for the analysis of prevalence data and that being our primary objective, this particular study design was chosen.

Study population

A prevalence study for any factor is best done in the community – in a lot of situations that would reduce the bias of a hospital study. This being a hospital study has its own limitations. However as far as possible we wanted to choose a population in our hospital survey that would most closely reflect the community or the general population at large. Hence for our study, we excluded people who were significantly ill or people with multiple interacting problems. We also excluded the elderly as in that population there would be the elements of age, poor absorption and poor nutrition confounding the results. Thus we ended up with the following:

Inclusion criteria:

1. Vegetarian patients coming to the out patient department of the Internal Medicine Department of Christian Medical College, Vellore, India.
2. Consenting males and females between 18 and 60 years of age.
3. First visit to Christian Medical College, Vellore.
4. Ambulant patient.

Vegetarian patients were defined as patients that had been consuming a diet devoid of any form of meat, at least for three completed years prior to the date of recruitment.

Exclusion criteria:

1. Patients on supplementation with vitamin B12 either orally or parenterally.
2. Patients with overt features of malabsorption.
3. Patients with chronic diarrhoea.
4. Patients with severe hepatic, renal, pulmonary, cardiac or neurological disease.
5. Patients with advanced malignancy.
6. Patients with dementia.

Study setting

This study was conducted in the out patient department of the Department of Internal Medicine, in Christian Medical College and Hospital, Vellore, which is a tertiary care teaching and research hospital, situated in Tamil Nadu, in South India. The biochemical analysis of the results was done in the Department of Clinical Biochemistry, in Christian Medical College and Hospital, Vellore, while the haematological parameters were measured at the Department of Clinical Pathology, in Christian Medical College and Hospital, Vellore.

Subject enrollment

Eligible patients, after fulfilling the inclusion and exclusion criteria, were informed about the nature of the study and their specific role in it and were recruited at

the time of first presentation to the out patient department, in the Department of Internal Medicine. Informed consent was taken, with the help of a form (see Annexure 1, "Informed Consent").

Methodology

A history and physical examination was done for all patients at the time of the first contact and enrollment. Detailed collection of data was performed for all the subjects in the following areas:

1. Epidemiological and geographic profile
2. Demographic profile and the reason for attending the outpatient clinic
3. Dietary profiles, that were sub divided into three groups
4. Clinical profile including specific symptoms and signs:
    a. Pallor
    b. Jaundice
    c. Hyperpigmentation
    d. Glossitis
    e. Sub acute combined degeneration
    f. Presence of paraesthesias
5. Comorbidity profile:
    a. Diabetes mellitus
    b. Hypertension
    c. Ischemic heart disease
    d. Cerebrovascular disease

6. Specific risk factors assessment – nature of drinking water, toxins, drugs exposure

7. Awareness profile – regarding this condition

8. Neuropsychiatric profile – in terms of anxiety and depression scores

9. Laboratory investigations:

a. Haemoglobin (by automated coulter counter)

b. Mean corpuscular volume (by automated coulter counter)

c. Serum vitamin B12 level (by chemiluminiscence assay)

d. Serum folate level (by chemiluminiscence assay)

e. Pernicious anaemia antibody screen

Data regarding the epidemiologic profile, the dietary profile, the clinical profile and the level of awareness were recorded in a preformed proforma (see Annexure 2, "Raw data collection sheet"). The proforma was filled in by the investigator at the time of the first interview and contact.

The neuropsychiatric profile of the subjects was assessed by two investigator administered questionnaires:

[1] Hospital Anxiety and Depression Score – This has been validated (70-73) before in various situations (Annexure 3).

[2] General Health Questionnaire – 12 (Annexure 4); this has also been validated before (74; 72).

Both the above two questionnaires could have been used as self administered forms, but for the sake of standardization, they were administered by the investigator who was constant for the entire population of study.

At the point of contact with the subject in the outpatient department he or she also underwent certain biochemical and haematological tests that included serum vitamin B12 and folate levels, and basic haemograms. For a subset of the subjects, serum pernicious anaemia antibody screens were also ordered for – they included the intrinsic factor antibody and the parietal cell antibody.

Sample size calculation

This study was a cross sectional prevalence study and hence the following expression was used to calculate the sample size.

$$n = \frac{z^2 p (1-p)}{d}$$

Where n = sample size, z = z statistic for a level of confidence, p = expected prevalence or proportion and d = precision.

According to existing data from the study by Yajnik et al (15) the prevalence of vitamin B12 deficiency was taken to be 60% and the precision was taken as 10%. With the above figures a sample size of 94 was arrived at. The aim of the study was to get a total of 100 people as the sample population. In total at the end of the study a total of 108 subjects were recruited and their results were analysed.

Biochemical estimation of vitamin B12

Vitamin B12 in the serum samples was measured by an Elecsys 2010 Vitamin B12 assay (MODULAR ANALYTICS 2010). The Elecsys vitamin B12 assay employs a competitive test principle using intrinsic factor specific for vitamin B12. Vitamin B12 in

the sample competes with the added vitamin B12 labelled with biotin for the binding sites on the ruthenium-labelled intrinsic factor complex.

Data integration and analysis

Data integration was done in the following main fields

1. Analysis of the prevalence of vitamin B12 deficiency in the study population.
2. Descriptive analysis of clinical manifestations of vitamin b12 deficiency.
3. Analysis of risk factors for vitamin B12 deficiency.
4. Descriptive analysis of the level of awareness amongst the study population.
5. Descriptive analysis of the degree of anxiety and depression within the study population using various scales of measurement.
6. Analysis of correlations between various groups.
7. Subgroup analysis amongst subjects with pernicious anaemia antibody screens.

Statistical analysis

Data entry was done using the Statistical Package for the Social Sciences (SPSS) software package (version 16). Descriptive statistics were tabulated using the SPSS software. The chi-square test was used for comparison of categorical variables. Odds ratios (OR) and confidence intervals (CI) were calculated and a ‘p’ value less than 0.05 was considered statistically significant. All reported p values are two-sided. Continuous variables were handled with the help of the student t tests and ANOVA tests.

RESULTS

A total of 118 subjects were interviewed of which a total of 10 subjects were excluded owing to the fact that they were on vitamin B12 supplementation. Hence a total of 108 subjects were included in the analysis.

Demography

Age

We recruited a total of 108 patients within the age cut-offs of 18 years and 60 years. The mean age of the study population was around 41 and half years. The age distribution is shown below (refer figure 7).

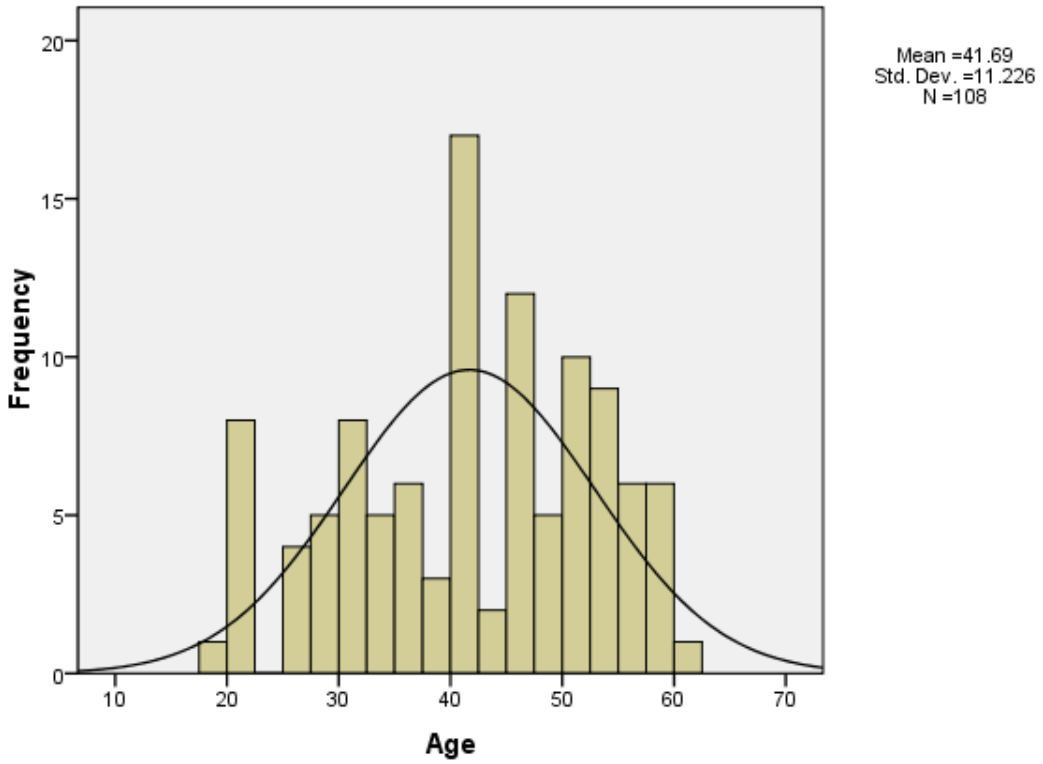

Figure 7: Distribution of age within the study population

Table 3: Age distribution in the population

Age groups (years)	**Numbers**	**Percentages**
Less than 20	6	5.6
21-30	16	14.8
31-40	25	23.1
41-50	30	27.8
51-60	31	28.7

Gender

The gender distribution was also uniform. 51.9% of the population were women and 48.1% were men.

Region of residence

The subjects in this study hailed from almost all over the country. The distribution according to the state of residency is shown below. Overall 78.7% of the study population was from north India and the remaining 21.3% was from south India – this is the general trend in our hospital where we see a major referral bias especially from the north of the country (refer figure 8).

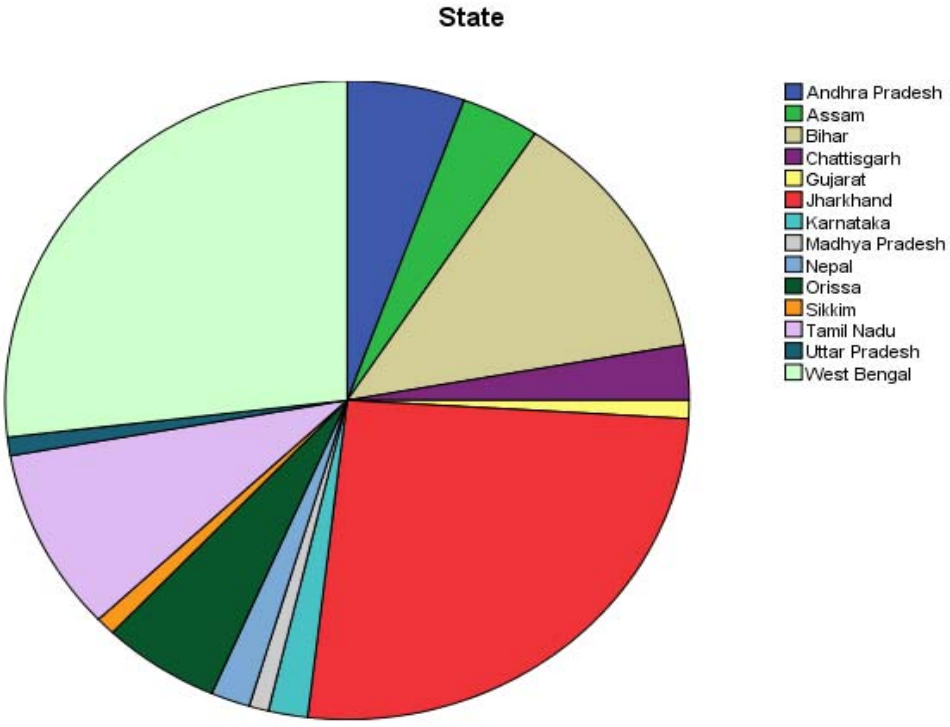

Figure 8: Distribution of the study population according to the state of residence

Table 4: Distribution of the study population according to the state of residence

	Frequency	Percent
Andhra Pradesh	6	5.5
Assam	4	3.7
Bihar	14	12.9
Chattisgarh	3	2.7
Gujarat	1	0.9
Jharkhand	28	26
Karnataka	2	1.8
Madhya Pradesh	1	0.9
Nepal	2	1.8
Orissa	6	5.5
Sikkim	1	0.9
Tamil Nadu	10	9.2
Uttar Pradesh	1	0.9
West Bengal	29	26.9
Total	108	100

Occupation

Though there were people from all wakes of life (refer table 5) most of the men were businessmen and most of the women were housewives in our study population.

Table 5: Various professions of our subjects

	Frequency	Percent
Accountant	1	0.9
Businessman	33	30.7
Chartered Accountant	1	0.9
Clerk	1	0.9
Contractor	1	0.9
Electrical engineer	1	0.9
Farmer	1	0.9
House wife	46	42.8
Maid	1	0.9
Pharmacist	1	0.9
Professor	2	1.8
Serviceman	3	2.8
Social worker	1	0.9
Software engineer	2	1.8
Staff nurse	1	0.9
Stock market trader	1	0.9
Student	8	7.5
Tata Steel Manager	1	0.9
Teacher	2	1.8
Total	108	100

Diet

We had stratified the dietary practices of the population into three groups:

a. Those who were pure vegetarians

b. Those who were vegetarians and consumed milk products as well

c. Those who were vegetarians and consumed milk products and eggs

The distribution of the above in our study population is as follows:

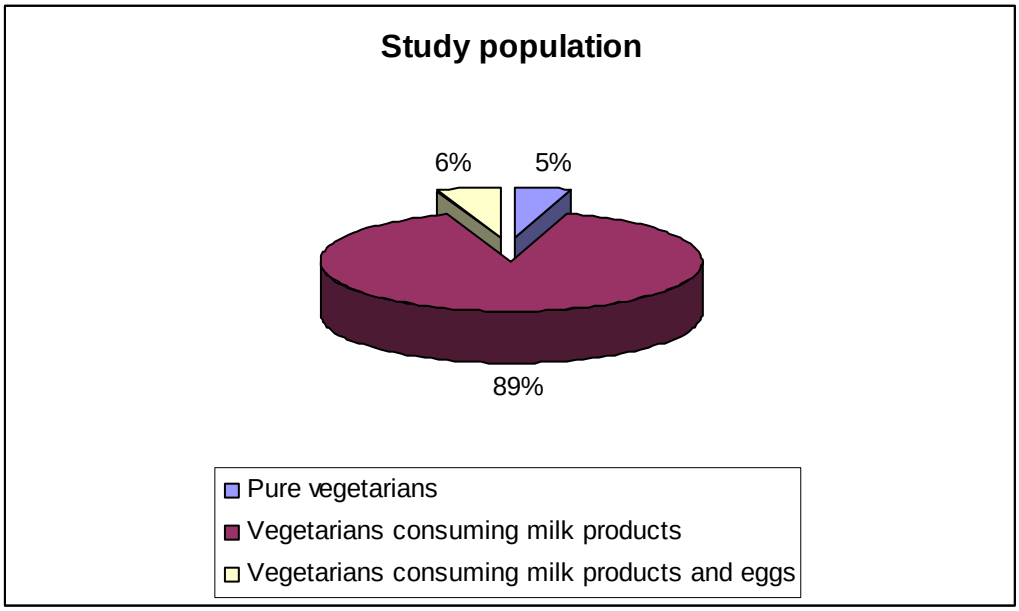

Figure 9: Dietary practices in the study population

Presenting complaints

In our exclusion criteria we had mentioned that people with major co morbidities pertaining to the major organ systems were to be excluded. Hence on analysis of the reason for presentation to the out patient clinic, we found that 61.1% had presented for the sake of a routine health check up without any specific ailments and 13% had presented with non specific chronic headache. Thus such a population could arguably be generalisable to the entire vegetarian community in India.

Prevalence of vitamin B12 deficiency

The characterisation of vitamin B12 deficiency was made according to well known standards (75-77). The following three categories were looked at:

* >300 pg/mL (>221 pmol/L) — normal result; cobalamin deficiency is unlikely (i.e., probability of 1 to 5 percent)

* 200 to 300 pg/mL (148 to 241 pmol/L) — borderline result; cobalamin deficiency possible

* <200 pg/mL (<148 pmol/L) — low; consistent with cobalamin deficiency (specificity of 95 to 100 percent)

The distribution of vitamin B12 in our population was as shown in Figure 10.

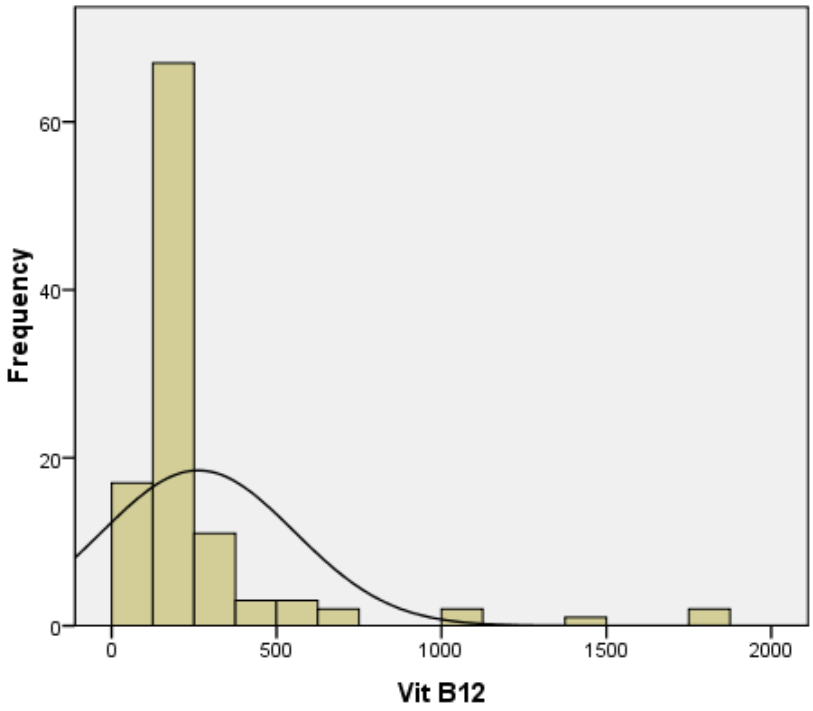

Figure 10: Distribution of vitamin B12 values within our study population

The mean vitamin B12 level in the population was 262.79 pg/mL and as we can see from figure 10, the vast majority of the subjects had vitamin B12 levels between 125 and 250 pg/mL.

In our study population, 61.1% had levels below 200, 23.1% had levels between 200 and 300 and 15.8% had levels above 300.

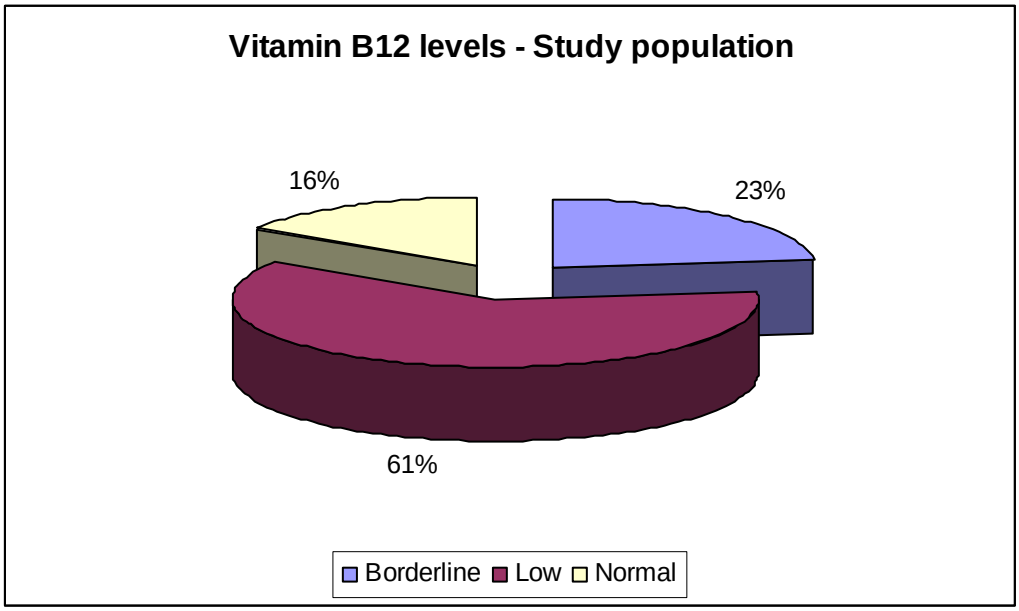

Figure 11: Vitamin B12 levels in the study population

The distributions were similar in the female and the male sub groups (refer table 6).

Table 6: Vitamin B12 categories among the genders

Vitamin B12 category	**Males – number(%)**	**Females – number(%)**	**Total**
Low	35(67.3%)	31(55.4%)	66(61%)
Borderline	7(13.5%)	18(32.1%)	25(23%)
Normal	10(19.2%)	7(12.5%)	17(16%)

Prevalence of folate deficiency

According to the biochemical kit for folate, available in our hospital and the reference values for that, any value between 3 and 17 ng/mL is considered normal. If we look at international reference standards the red cell folate is a better marker of long term

folate deficiency than the serum folate per say. However, a serum folate level between 2 and 4 ng/mL maybe considered as a borderline value. Only 3 out of our 108 subjects had a folate level less than 4, and none had a value less than 2. The mean serum folate level in our population was 9.97ng/mL and the distribution of the folate levels was as shown below in figure 12.

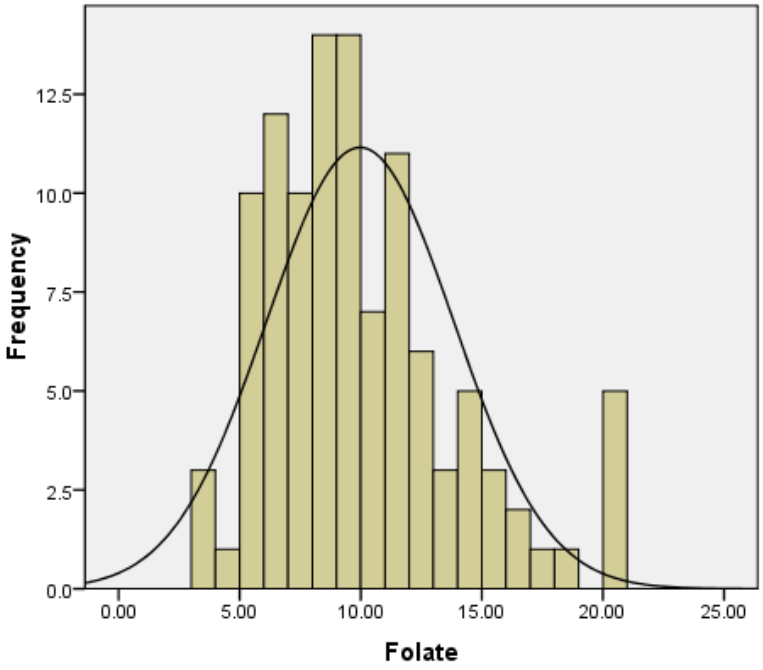

Figure 12: Distribution of folate levels in the study population

The mean values of folate in the low, borderline and normal vitamin B12 groups were 9.0, 9.9 and 13.6 ng/mL. The mean folate level was significantly different between the groups by one way ANOVA, with a p value of less than 0.001 (refer table 7). **Thus the mean folate concentration was significantly higher in the normal B12 group compared to the low B12 group.**

Table 7: Mean folate levels in the three B12 categories [P<0.001 (by ANOVA)]

					95% Confidence Interval for Mean	
		Mean folate level	Std. Deviation	Std. Error	Lower Bound	Upper Bound
Low B12	66	9.02	3.12	.384	8.25	9.78
Borderline B12	25	9.97	3.62	.725	8.47	11.47
Normal B12	17	13.66	4.70	1.141	11.24	16.08

There was also a statistically significant correlation between the serum folate levels and the vitamin B12 levels throughout the entire population, the Pearson's correlation coefficient being 0.314 (refer figure 13).

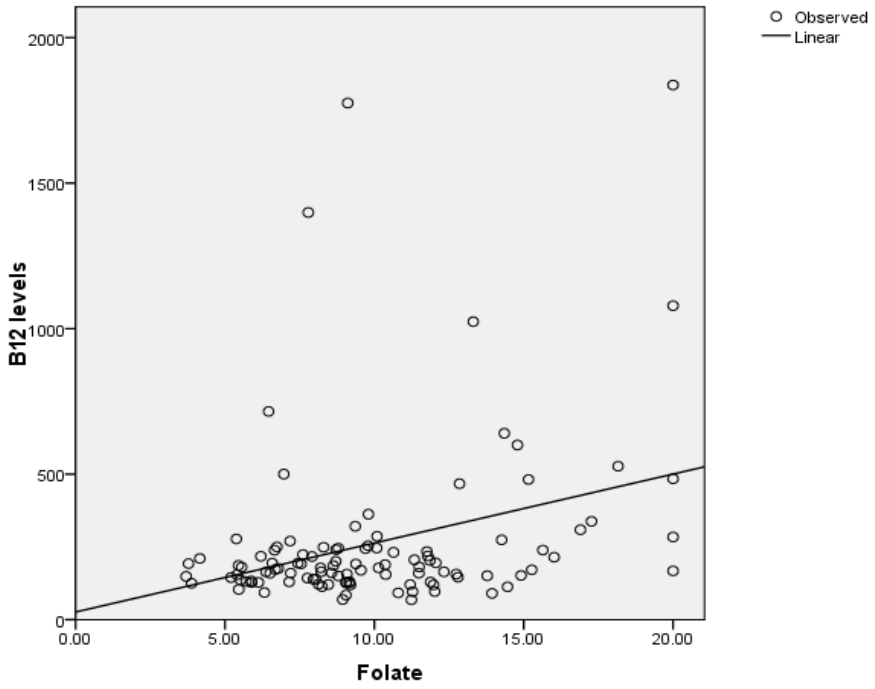

Figure 13: Correlation between folate and B12 values in the entire study population (p=0.001)

Hence as is clear from Figure 14, there was a clear correlation between the folate levels and the B12 levels that was statistically significant. Patients with lower B12 levels had lower folate levels.

Clinical profile

We looked at the clinical profile of the subjects with respect to the following parameters: (refer table 8)

Table 8: Prevalence of various clinical parameters in the three B12 categories

Clinical parameter	Low B12	Borderline B12	Normal B12
Hyperpigmentation	9.1%	12%	5.9%
Glossitis	45.5%	36%	41.2%
SCD	1.5%	0%	0%
Paraesthesias	42.4%	56%	35.3%
Diabetes	13.6%	8%	17.6%
Hypertension	28.8%	12%	17.6%

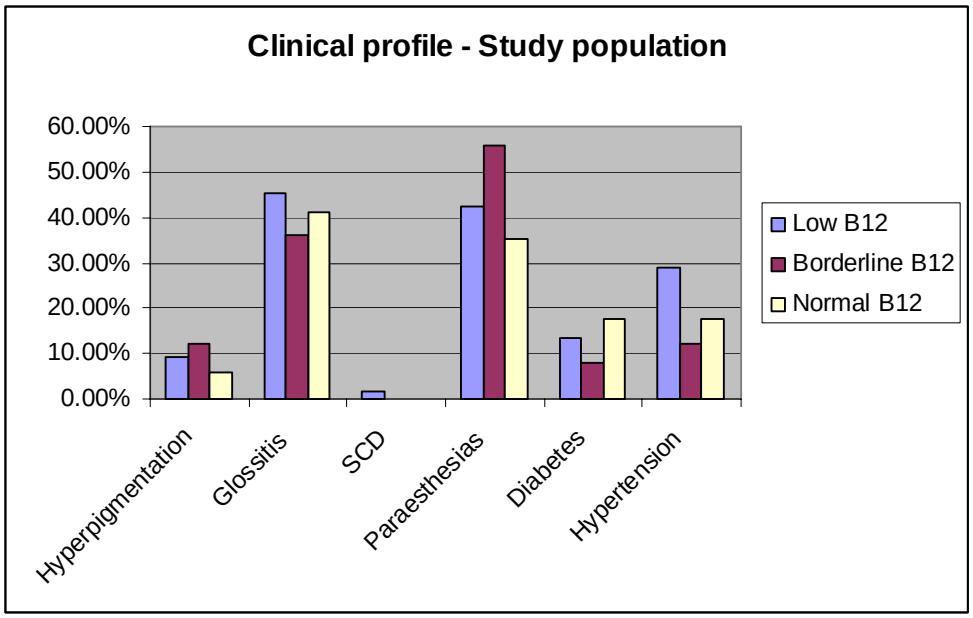

Figure 14: Comparison of clinical parameters among the B12 categories

Laboratory profile

We had also looked at a few laboratory parameters in the study population.

Haemoglobin

The mean haemoglobin value in our study population was 12.9 g/dL. The lowest haemoglobin value was 8.4 and the highest was 17. 29.6% of the population had a haemoglobin value of less than 12 and hence could be classified as anaemic.

In the low vitamin B12 group the mean haemoglobin level was 12.9 g/dL. 28.8% of these people had a haemoglobin value of less than 12.

In the borderline vitamin B12 group the mean haemoglobin level was 12.6 g/dL. 36% of these people had a haemoglobin value of less than 12.

In the normal vitamin B12 group the mean haemoglobin level was 13.2 g/dL. 23.5% of these people had a haemoglobin value of less than 12.

Table 9: Haemoglobin values in the three B12 groups

Parameter	Haemoglobin (average gm%)	Haemoglobin <12gm%: number(%)
Low B12 group	12.9 gm%	19(28.8%)
Borderline B12 group	12.6 gm%	9(36%)
Normal B12 group	13.2 gm%	4(23.5%)

Mean corpuscular volume

The distribution of mean corpuscular volume in the population was as shown in the next page (figure 15):

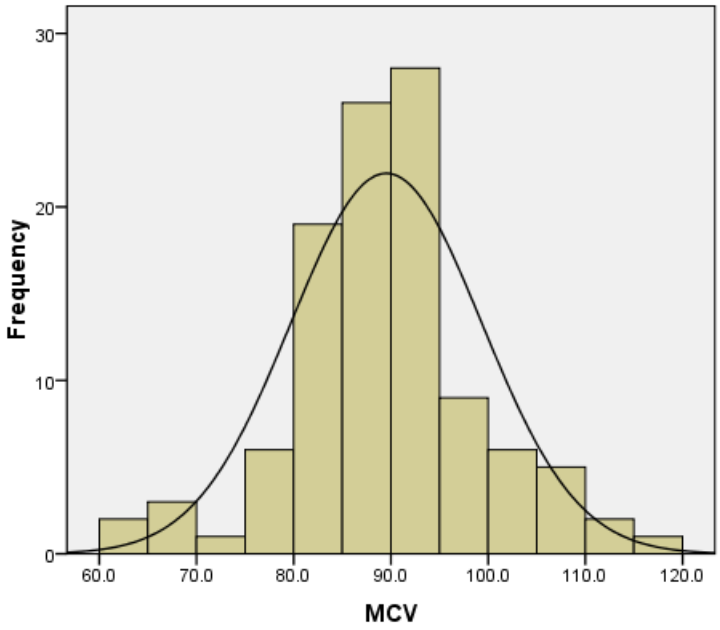

Figure 15: The distribution of mean corpuscular volume (MCV) in the study population

The mean MCV in the study population was 89.2 fl. The corresponding values in the low, borderline and normal B12 categories were 90, 87 and 89 fl, which were no different from each other statistically. 19.7% in the low B12 group as opposed to 4% in the borderline B12 group had macrocytosis (refer figure 16).

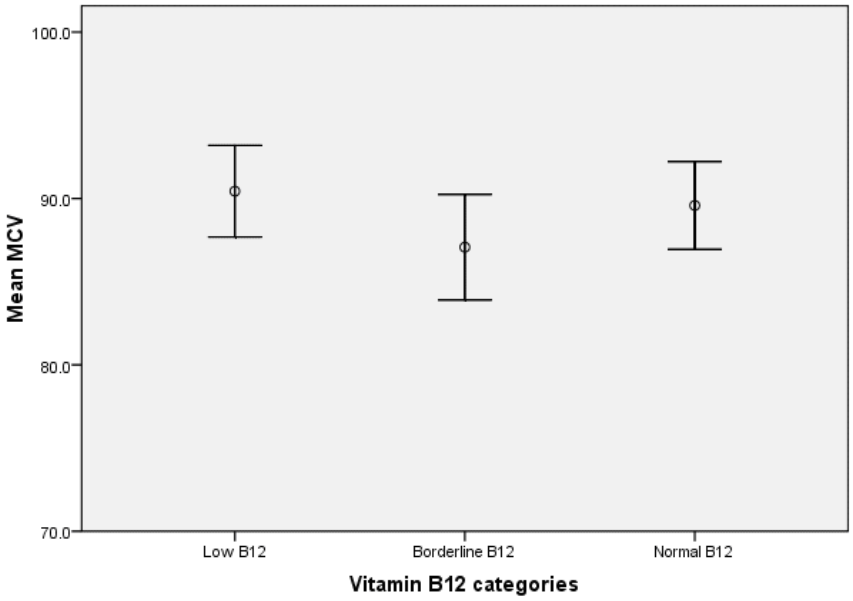

Figure 16: Mean MCV in the three B12 categories (p=0.38)

None of the subjects in the normal B12 group had macrocytosis. These values were significantly different between the groups by the Chi square test with a p value of 0.031 (refer table 10).

Table 10: Mean corpuscular volumes in the three B12 categories (p=0.031)

Parameter	Average MCV fl	Number (percentage) of patients with macrocytosis (MCV >100 fl)
Low B12 group	90	13 (19.7%)
Borderline B12 group	87	1(4%)
Normal B12 group	89	0(0%)

Hence macrocytosis was significantly associated with B12 deficiency and indicated the presence of this condition(p=0.031).

Microcytosis

In the study population we looked for microcytosis and we found that 11.1% of the subjects actually met the definition of microcytosis, which is a mean corpuscular volume less than 80 fl (78). Strangely enough, all the microcytosis was in the low and borderline vitamin B12 categories with no microcytosis at all in the normal vitamin B12 category. Statistically however there was no significant differences between the groups (refer table 11).

Table 11: Microcytosis amongst the different B12 categories (p= 0.276)

	Microcytosis		Total
	Present	Absent	
Borderline B12	3(12%)	22	25
Low B12	9(13.6%)	57	66
Normal B12	0(0%)	17	17
Total	12(11.1%)	96	108

Predictors of vitamin B12 deficiency

One of the objectives of our study was to look for predictors or associations of vitamin B12 deficiency. In that regard we studied a few parameters, handling each of them in the statistically appropriate methods.

Age

We looked at age to see whether it was significantly associated with vitamin B12 deficiency. There was no significant difference in the mean ages of the subjects amongst the three vitamin B12 categories by ANOVA (p=0.102).

Diet

We had classified the subject population into three categories: pure vegetarians (vegans), vegetarians who consumed milk products and vegetarians who consumed milk products and eggs (eggeterians). The mean vitamin B12 levels in the three groups respectively were 145, 270 and 230. There was no statistically significant difference between the groups by ANOVA (refer figure 17).

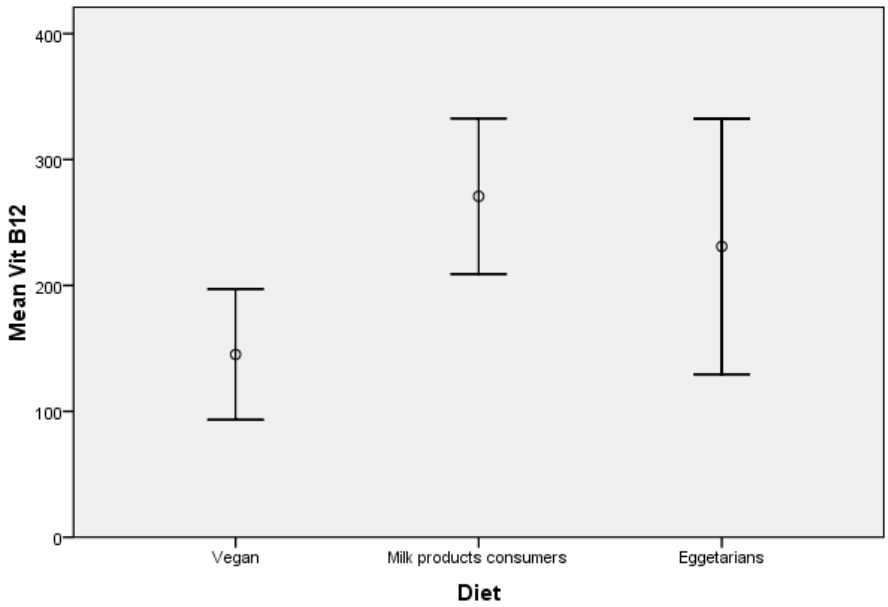

Figure 17: Mean vitamin B12 levels amongst the three diet categories (p=0.623)

We also looked at various laboratory parameters among the three varieties of diet consumers – however the numbers were too small for any statistical analysis between them (refer table 12).

Table12: Laboratory parameters amongst the three dietary groups

Parameter	Pure vegetarian (N=5)	Vegetarian with milk products (N=97)	Vegetarian with milk products and eggs (N=6)
Average B12 value	145.23	270.82	230.98
Low B12	4(80%)	58(59.8%)	4(66.7%)
Average MCV	93.44	89.20	91.38
Macrocytosis	2(40%)	11(11.3%)	1(16.7%)
Average Hb	13.12	12.92	12.96
Anaemia (Hb<12)	1(20%)	28(28.9)	3(50%)

Odds ratios and Chi-square tests

We used odds ratios and Chi square tests for studying categorical variables (refer table 13) as are shown below. For this analysis we assumed people who had vitamin B12 levels below 200 to be in the "low" vitamin B12 category and all the others to be in the "normal" B12 category. The "borderline' category was not used, both for statistical problems and small numbers as well as for the fact that the concept of "borderline" vitamin B12 levels is neither very uniform not very well characterised in other studies.

Mean corpuscular values of less than or equal to 100fl were categorised as normocytic category and anything higher than that was considered as qualifying for macrocytosis (43). A folate level cut-off at 9.5 was also taken to look for prediction of vitamin B12 deficiency.

In our study we had classified the type of drinking water into seven types. For the sake of analysis and small numbers we grouped them into "pure water" that included

mineral water, aquaguard water and boiled water and “less pure water” that included all the rest of the categories for purity of water (tap water, well water, filter water and chlorinated water).

Table 13: Odds ratios of clinical parameters

Parameter		Numbers (Percentages)		Odds ratio	Confidence intervals	P
		Low B12 group	Borderline and normal			
Gender	Male	35(67.3%)	17(22.7%)	1.66	0.759-3.633	0.203
	Female	31(55.3%)	25(44.7%)			
Region	North	51(60%)	34(40%)	0.8	0.306-2.093	0.649
	South	15(65.2%)	8(34.8%)			
Hyperpigmentation	Present	6(60%)	4(40%)	0.95	0.252-3.588	0.94
	Absent	60(61.2%)	38(38.8%)			
Glossitis	Present	30(65.2%)	16(34.8%)	1.354	0.615-2.981	0.451
	Absent	36(58.0%)	26(42.0%)			
Paraesthesias	Present	28(58.3%)	20(41.7%)	0.811	0.372-1.764	0.596
	Absent	38(63.3%)	22(36.7%)			
Betel use	Present	10(66.6%)	5(33.4%)	1.321	0.418-4.178	0.634
	Absent	56(60.2%)	37(39.8%)			
Jarda use	Present	8(61.5%)	5(38.5%)	1.021	0.310-3.359	0.973
	Absent	58(61.0%)	37(39.0%)			
PPI use	Present	3(37.5%)	5(62.5%)	0.352	0.080-1.560	0.155
	Absent	63(63%)	37(37%)			
H2RB use	Present	3(75%)	1(25%)	1.952	0.196-19.417	0.561
	Absent	63(60.5%)	41(39.5%)			
Metformin use	Present	4(57.1%)	3(42.9%)	0.839	0.178-3.950	0.824
	Absent	62(61.3%)	39(38.7%)			
MCV	>100	13(92.8%)	1(7.2%)	10.057	1.263-80.048	0.009
	<100	53(56.3)	41(43.7%)			
Folate	<9.5 ng/mL	43(71.6%)	17(29.4%)	2.749	1.238-6.103	0.012
	>9.5 ng/mL	23(47.9%)	25(52.1%)			

Water

We had collected information as to the various sources of drinking water for the subjects, to see if the type of water or the purity had any bearing on the levels of vitamin B12. The individual numbers in each of the categories of drinking water were very small – however on grouping them as mentioned above into two categories "less pure water" and "pure water", there were no statistically significant odds of predicting a vitamin B12 deficiency (refer table 14).

Table 14: Odds ratio for the type of water: 1.169, 95%Confidence interval: 0.539-2.535, p=0.693

Parameter	Low B12 group	Borderline and normal
Less pure water	34(62.9%)	20(37.1%)
Pure water	32(59.2%)	22(41.8%)

Logistic regression

On the basis of the above we did a logistic regression to see which of the above factors remain significant on a multivariate analysis. We found that only the mean corpuscular volume retains significance on a multivariate model, with a p value of 0.048 (refer table 15).

Table 15: Logistic regression

	Std Error	Deg of freedom	P value	Risk
Age	0.01	1	0.59	
Sex	0.42	1	0.25	
Folate level	0.43	1	0.11	
MCV	1.07	1	**0.04**	**8.4**
PPI	0.84	1	0.2	

Hence, on a multivariate analysis, a patient with macrocytosis (MCV>100fl) had an 8 fold risk of B12 deficiency compared to a patient with normocytosis.

Awareness regarding vitamin B12 deficiency

As a part of the clinical interview the subjects had been asked three questions to assess the level of their awareness regarding vitamin B12 deficiency (refer Annexure 2). Only 4 people out of the 108 that were finally included in the analysis, were actually aware of a condition known as vitamin B12 deficiency. Only one of them actually knew that being a vegetarian made them prone to vitamin B12 deficiency, and only that same person knew that vegetarians might actually have to take supplemental vitamins to prevent vitamin B12 deficiency. Hence the awareness regarding this condition is extremely poor, almost to the extent of being non existent.

Anxiety and depression studies

During the clinical interview we had done an assessment of the prevalence of anxiety and the depression within the study population with the help of two widely used scoring systems – namely the General Health Questionnaire – 12, and the Hospital Anxiety and Depression Score. Summarised below are the results.

General Health Questionnaire – 12 (GHQ-12)

The GHQ-12 theoretically can have scores between 0 and 36, with scores upto 15 being taken as normal, 16-20 suggesting evidence of distress and 21 and above signifying severe distress.

The mean GHQ-12 score in our population was 17.70 and the distribution of the scores is as below.

The mean GHQ-12 scores in the low, borderline and normal B12 categories were 17.62, 18.00 and 17.59 respectively. Percentages of subjects with GHQ-12 scores signifying normal score, evidence of distress and severe distress among the three B12 categories are shown below (refer figure 18).

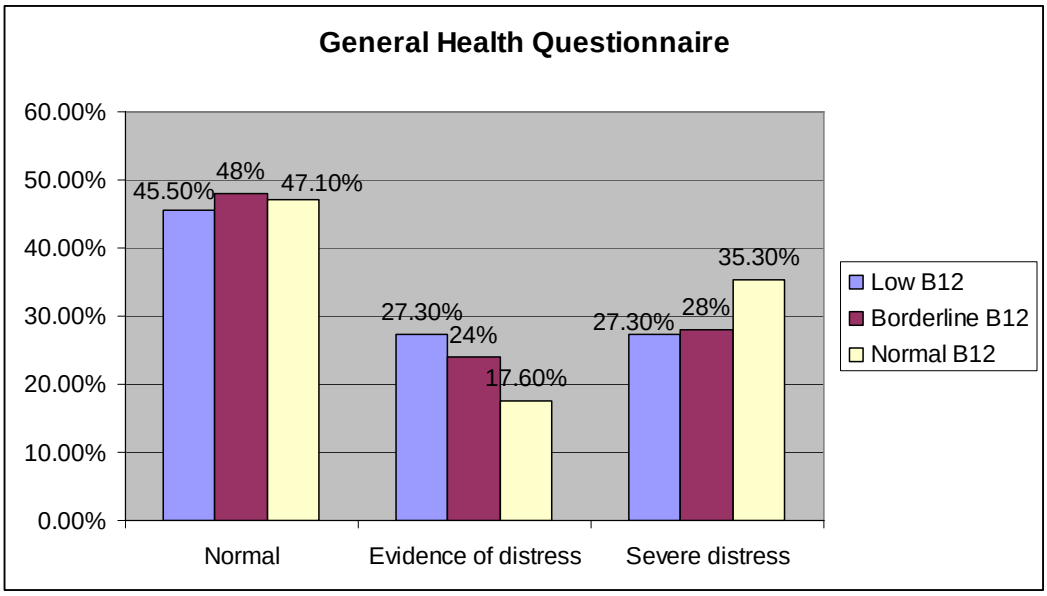

Figure 18: GHQ-12 grades in the three B12 categories

Overall, in the entire study population, the mean vitamin B12 levels in the three GHQ-12 categories namely “normal”, “evidence of distress” and “extreme distress” were 258.79, 256.76 and 274.49. There was no statistically significant difference between the groups (p=0.966 by ANOVA).

We also looked at whether the GHQ-12 scores correlated with the vitamin B12 values within the entire study population – there was no such significant correlation (refer figure 19).

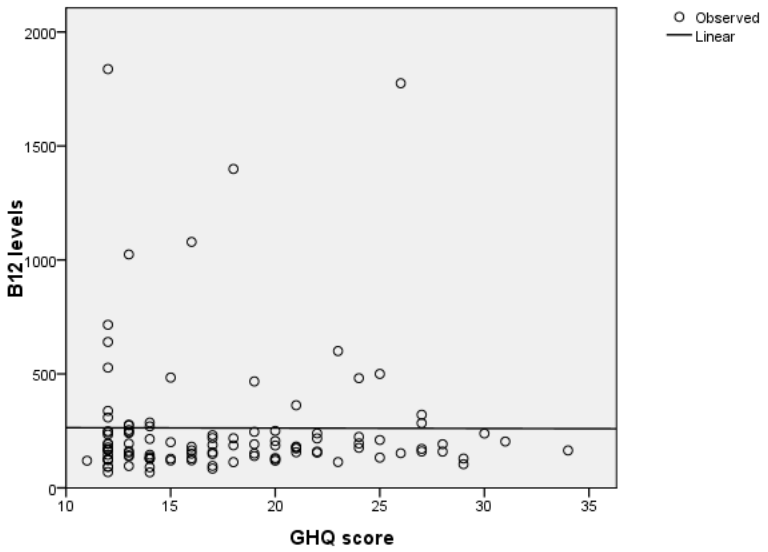

Figure 19: Correlation between GHQ-12 scores and B12 levels in the entire population (p=0.973)

Hospital Anxiety and Depression Score – Anxiety (HADS-A)

The HADS-A theoretically can have scores between 0 and 21, with scores upto 7 being taken as normal, 8-10 suggesting borderline abnormal and 11 and above signifying abnormal scores.

The mean HADS-A score in our population was 7.45 and the distribution of the scores is as below.

The mean HADS-A scores in the low, borderline and normal B12 categories were 7.77, 7.76 and 5.76 respectively. Percentages of subjects with HADS-A scores signifying

normal , borderline abnormal and abnormal scores, among the three B12 categories are shown in Figure 20.

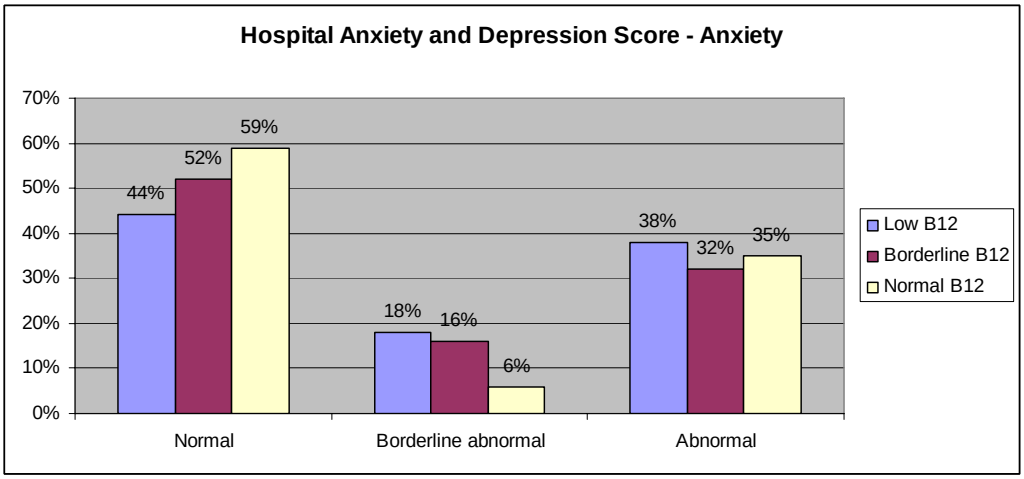

Figure 20: HADS - A grades in the three B12 categories

Overall, in the entire study population, the mean vitamin B12 levels in the three HADS-A categories namely “normal”, “borderline abnormal” and “abnormal” were 278.89, 237.53 and 252.33. There was no statistically significant difference between the groups (p=0.847 by ANOVA).

We also looked at whether the HADS-A scores correlated with the vitamin B12 values– there was no such significant correlation (refer figure 21).

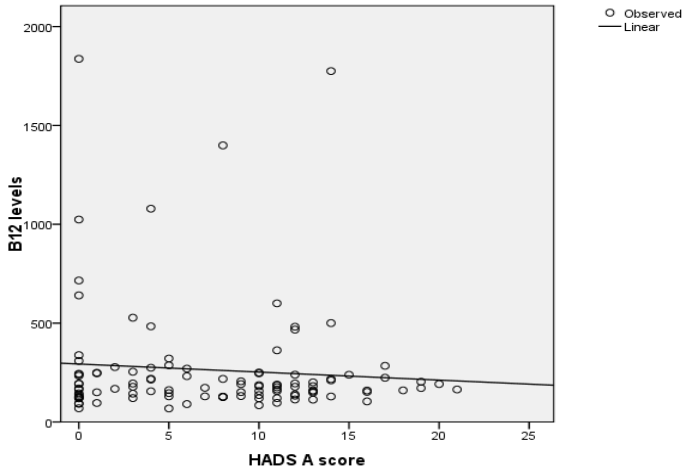

Figure 21: Correlation between HADS-A scores and B12 levels (p=0.402)

Hospital Anxiety and Depression Score – Depression (HADS-D)

The HADS-D theoretically can have scores between 0 and 18, with scores upto 7 being taken as normal, 8-10 suggesting borderline abnormal and 11 and above signifying abnormal scores. The mean HADS-D score in our population was 3.84 and the distribution of the scores is as below.

The mean HADS-D scores in the low, borderline and normal B12 categories were 3.92, 3.96 and 3.35 respectively. Percentages of subjects with HADS-D scores signifying normal, borderline abnormal and abnormal scores, among the three B12 categories are shown below.

Overall, in the entire study population, the mean vitamin B12 levels in the three HADS-D categories namely "normal", "borderline abnormal" and "abnormal" were 260.5, 292.01 and 207.10. There was no statistically significant difference between the groups (p=0.819 by ANOVA).

We also looked at whether the HADS-D scores correlated with the vitamin B12 values within the entire study population – there was no such significant correlation (refer figure 23).

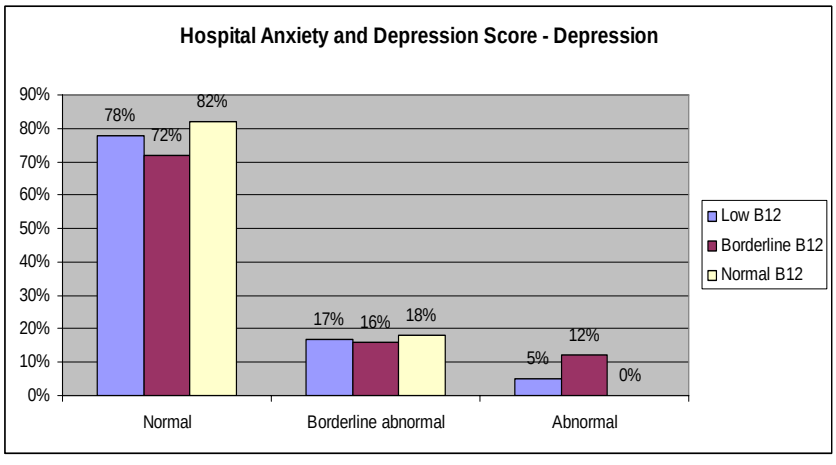

Figure 22: HADS-D grades in the three B12 categories

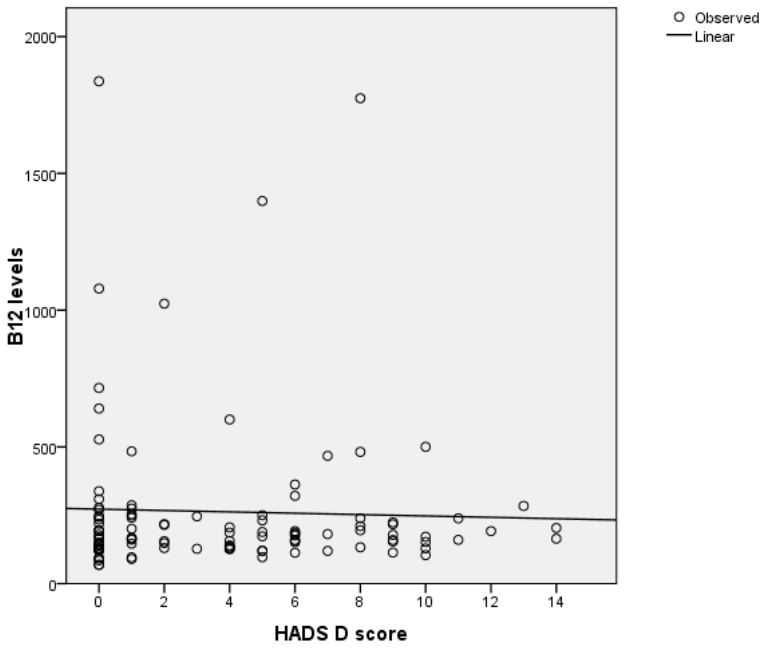

Figure 23: Correlation between HADS-D scores and B12 levels in the entire population (p=0.732)

Hence none of the depression or anxiety scores showed any correlation between the levels of distress and the levels of vitamin B12.

Pernicious anaemia antibody screens

We had performed pernicious anaemia antibody screens amongst a small sub group of our subjects. In total we did 23 pernicious anaemia antibody screens (17 amongst vitamin B12 deficient and 6 amongst vitamin B12 non deficient, i.e. with B12 levels above 200). In total we had 10 positive screens and 13 negative screens. Intrinsic factor antibody was positive in 4 cases, parietal cell antibody was positive in 4 cases and both were positive in 2 cases. 47% of the pernicious anaemia antibody screens were positive in the low B12 group as opposed to 33% in the normal group. Owing to the small

numbers and the nature of the sub group and the uncertainty of random sampling no statistical analysis could be performed with this data (refer table 16).

Table 16: Pernicious anaemia antibody screens

Pernicious anaemia antibody screens versus Vitamin B12 levels				
Count				
		Vitamin B12 levels		
		Low	Normal	Total
Pernicious anaemia antibody(PAA) screen	Positive	8	2	10
	Negative	9	4	13
Total		17	6	23

DISCUSSION

This was a cross-sectional study among vegetarians between the age groups of 18 and 60 years visiting the out patient department of a tertiary level hospital in South India.

Population profile

We recruited 108 patients in all, that hailed from all over the country. People from the north were more than those from the south – a trend quite common in our hospital owing to the referral bias. Men and women were almost equal in number and the overall age distribution appeared uniform. All subjects were Hindus. We had excluded the elderly age group and the paediatric age group. Owing to this being a hospital based study with all its inherent biases the question of generalisability to the community at large is debatable. On the other hand we had excluded patients with any major co morbidity and analysis showed that 61.1% of people had come for a health check up with no specific complaints. Only 13% had diabetes and 23% had hypertension. In view of the above, the selected population appears fairly well and fit, akin to the expected selection in a community based population – making the possibility of extrapolating such a result to the community setting, acceptable.

Prevalence of vitamin B12 deficiency

The prevalence of vitamin B12 deficiency (level less than 200), amongst overall healthy patients presenting mainly for a check up was 61%. Borderline B12 levels (between 200 and 300), were seen in 23% more people. Hence 84% of the population had levels that were either borderline or low, and only 16% were absolutely normal. This is

comparable to the community study by Yajnik et al (15), in which 67% of the population had a low B12 level.

Hence from this data it is clear that absolutely low levels of vitamin B12 among vegetarians, are rampant in the country, and the percentages rise even higher if we look at borderline B12 levels. We know from previous studies that even with low normal B12 levels (i.e. in the borderline levels) there are biochemical changes that initiate within the body that can potentially be damaging. Hence almost 84% of the population studied here are at a risk for possible end organ damage. This is a large number and it remains to be seen if the same trend is being duplicated in other parts of the country as well.

Mean corpuscular volume

Macrocytosis was significantly more frequent in the low vitamin B12 category. 13% of the population had an MCV of 100fl or more (19% amongst deficient people, 4% amongst borderline B12 people and none amongst people with normal B12 levels).This is starkly in contrast to Yajnik's study (15), where only 2% of the study population had macrocytosis. Also 11.1% had an MCV of less than 80fl, which is the classical cut-off for indicating microcytosis (78). There might have been a sizeable proportion within our study population that may have had a subclinical iron deficiency leading to a dimorphic blood picture that might have skewed the percentage of macrocytosis. Interestingly 13.6% of the subjects in the low B12 category and 12% in the borderline B12 category had microcytosis – whereas in the normal B12 category there were absolutely no subjects with microcytosis. This only strengthens the possibility of coexisting sub-clinical iron deficiency in the low and borderline B12 categories. Iron studies and ferritin may have

been useful parameters for monitoring in this situation, that could be done in subsequent studies. Malnutrition contributing to the above would be unlikely, as most of the subjects were from a well to do socioeconomic background. In view of the above possibility of coexisting sub-clinical iron deficiency that simply cannot be attributed to a vegetarian dietary intake, malabsorbtive syndromes could be a clinical differential in this subgroup of people that needs to be clinically explored.

Prevalence of folate deficiency

Only three out of our 108 subjects had a folate level of less than 4 and none had a level below 3. Hence effectively there was almost zero prevalence of folate deficiency in our study population. Folate deficiency may not always coexist with B12 deficiency, their physiology and metabolism being distinct. Serum folate levels typically take very short whiles to normalise – for example a single meal maybe enough to normalise serum folate even if tissue stores are low. Hence it has been argued before that red cell folate is a better indicator of tissue folate stores. Furthermore folate is obtained from greens and not solely obtained from animal products. Folate deficiency is typically seen in subjects who are chronic alcoholics and those who are on medications that might antagonise folate metabolism – none of those scenarios where prevalent in our study population. Hence it was not surprising that we did not pick up any folate deficiency.

The mean folate level in our population was 9.97ng/mL and the distribution was uniform. Within the normal levels of folate, there seemed to be a distinct correlation between the folate levels and the B12 levels, and a folate level less than 9.5 predicted B12 deficiency on a univariate analysis. The significance of this finding is not clear,

though the possibility of a malabsorptive disorder (given that associated iron deficiency and microcytosis probably also exists in this population), might be considered to explain the observation.

Clinical profile

Glossitis, paraesthesias and pallor where the most common clinical features amongst the subjects with low vitamin B12 levels. Hyperpigmentation and jaundice where seen to be less frequent in the B12 deficient group. Only one patient in the low B12 category had clinical features commensurate with a diagnosis of subacute combined degeneration of the cord. No clinical feature could actually predict low B12 levels in the population. Diabetes and hypertension were not very frequent among the groups. Nobody had any other major co morbidity. Psychiatric symptoms have been analysed separately in a later section.

Haemoglobin

Overall the study group looked well preserved with a mean haemoglobin value of 12.9 g/dL. Mean haemoglobin levels in the three B12 categories appeared to be similar. The population haemoglobin distribution also appeared Gaussian. About 29% had haemoglobin levels below 12. This is again another point to indicate that the population chosen was not majorly diseased as would be expected in a hospital population survey – indicating that probably the results could be extrapolated to the community. In the study by Yajnik et al (15) the quoted figures for anaemia (in that study haemoglobin less than 13.5 had been defined as anaemia), were comparable to ours.

Predictors or associations of vitamin B12 deficiency

We studied various clinical parameters, drug exposure, water source and biochemicals to see whether the presence of any of them could significantly predict vitamin B12 deficiency. The univariate analyses have been given in a previous section. There were not many people in our study population who had been exposed to the drugs that we studied, hence probably the numbers were not really adequate. On the univariate analysis only two factors namely an MCV of greater than 100fl and a folate level of less than 9.5ng/mL significantly predicted a vitaminB12 level of less than 200. When we adjusted the risk ratios for various coviariates using logistic regression, only an MCV of greater than 100fl actually predicted a vitamin B12 level of less than 200 – all the rest of the factors were insignificant on the multivariate analysis. This may not be the true picture in terms of risk factor analysis principally for two reasons – firstly this study was not really powered towards looking into causal associations and secondly the numbers in each of these subgroups were probably not large enough to achieve statistically significant results.

Awareness

An extremely low level of awareness of the vegetarian population under study, regarding this condition of vitamin B12 deficiency, was clearly apparent from the analysis of the questionnaires. However it must be remembered that this study had excluded people (10 people to be exact) on any kind of vitamin supplementation, and that excluded population might have had a better awareness of the condition. To our knowledge this aspect has not been studied previously. Given the fact that the prevalence

of the condition is high and vegetarianism is common in India (6), it becomes all the more important to assess the awareness of this condition in the community, for public health benefits. There may be important clinical and therapeutic implications of this in the field of prevention of this condition. This maybe an important issue to look into, in future studies.

Anxiety and depression studies

One important aspect of this study was to look at the neuropsychiatric symptomatology in our study population. Though neuropsychiatric manifestations have been described in the B12 deficient, there is not much data as to the patterns and prevalences of these symptoms. With both our screening tools (i.e. the General Health Questionnaire – 12, and the Hospital Anxiety and Depression Score), we found a high level of anxiety and depression in our study population. Unfortunately there were no major statistical differences between the low, borderline and normal B12 categories in terms of characterization and degree of neuropsychiatric symptoms – none of them were found to be able to significantly predict B12 deficiency. Correlation studies between the anxiety and depression scores and the B12 levels in the population were not found to be statistically significant. The cause for this negative result is probably three things:
A] Being a hospital cohort the general level of anxiety among the study population would have been expected to be higher than in a community cohort (to keep in mind, most of the subjects had voluntarily come for check ups – making a point that a certain degree of anxiety would have triggered this overt health seeking behaviour)

B] The study was again not really powered to pick up differences in the neuropsychiatric manifestations between the three different B12 categories

C] Only screening tests were used to gauge neuropsychiatric symptoms – perhaps detailed DSM IV or ICD-10 criteria for anxiety and depression would be necessary to pick out statistically significant differences between the various groups

However overall, this study does tell us that the level of neuropsychiatric symptoms in this population is high, and would possibly be a pertinent point for further investigations in the future.

Pernicious anaemia antibody screens

Owing to various constraints we were able to perform pernicious anaemia antibody screens in only few limited numbers among our subjects. Of our 23 tests, 10 results were positive, of which 5 had positivity for intrinsic factor antibody. We did a univariate cross-tabulation between pernicious anaemia antibody screens and vitamin B12 levels in that small subgroup of 23 that did not reveal any statistically significant result. Numbers obviously were a major problem here. However this high level of positivity with the antibody screens brings us to the point of whether there is hidden pernicious anemia, or for that matter the autoimmune polyglandular syndromes, within this population. We understand that these antibodies have false positives and we have not done gastric biopsies for any of our patients, to claim anything very significant – but still given the unusually high rate of positivity for these markers, further investigations are probably of the order to probe deeper into this matter.

LIMITATIONS

There were a few important limitations to this study:

A] The hospital based population selection has its inherent biases and problems of extrapolation of the results to the community.

B] The design of the study being cross-sectional, analysis of risk factors and drawing up associations including causal associations might not be without its problems.

C] The small numbers may not have been adequate to bring out the statistical significances of certain studied factors.

D] The high rates of anxiety and depression may have been owing to the inherent selection bias of this hospital based cohort. The screening tools may also have contributed to this.

E] The analysis of pernicious anaemia antibody screens was not complete owing to various constraints.

CONCLUSIONS

This was a cross sectional study among vegetarian out patients where we found the following:

A] There was a high prevalence of vitamin B12 deficiency (61%) in the study population. Another 23% had borderline vitamin B12 levels. Most of them were not overtly symptomatic.

B] The most common clinical features were glossitis and paresthesias. Subacute combined degeneration of the spinal cord was rare.

C] Macrocytosis (MCV>100fl) was seen in 19.7% of B12 deficient patients and in 4% of the borderline B12 group. An MCV higher than 100fl and a folate level less than 9.5ng/mL predicted vitamin B12 deficiency. Also, the folate levels correlated very well with the vitamin B12 levels.

D] Etiology of vitamin B12 deficiency in the population is unlikely to be purely dietary and could be multi-factorial including malabsorption and pernicious anaemia.

E] Awareness about B12 deficiency was extremely low.

F] There was a high prevalence of neuropsychiatric symptoms in the entire study population – there were no significant differences between the B12 categories in terms of anxiety and depression scores.

G] Within a small subgroup of the study population, an unusually high level of pernicious anaemia antibody positivity was found, that could not be adequately handled statistically owing to small numbers.

Overall, there were a number of clinical and statistical possibilities that emerged from this small study, that require further investigation in larger studies in the future.

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Annexures

Annexure 1

INFORMED CONSENT

This is a study that is being done to look at the prevalence of Vitamin B12 deficiency and the clinical manifestations and the risk factors associated with this condition.

Should I agree to participate in this study I will have to undergo the following:

a. When I come to the OPD for the very first time I will be asked some questions, some about my symptoms and some about things like the type of water I use, whether I chew pan, whether I take any other medications etc.
b. Following this I will have a physical examination.
c. Following this I will be required to fill a general health questionnaire.
d. Following this I will be given a few blood tests to do.

I will not have to undergo any special procedure if I am consenting for this study. I will not incur any extra costs for this and my treatment will be no different from any other person with a similar disease.

I understand that my clinical data will be available to all the people involved in the research but my name will be held confidential. Confidentiality of patient information will be maintained.

I understand that my participation is voluntary, and I have full right to withdraw from this study at any point without giving any reason.

In the event that I am detected to be Vitamin B12 deficient, I will be appropriately advised regarding the treatment for the condition.

I have read the contents of this page and have had ample opportunities to ask questions about it. I understand the contents of this page and I hereby agree to give my consent towards my participation in this study.

Signature of the subject:	Signature of the investigator:
Name of the subject:	Name of the investigator:
Date:	Date:

Annexure 2

RAW DATA COLLECTION SHEET

Name of the subject:

Age:

State:

Relegion:

Diet: pure vegetarian / vegetarian and milk products / vegetarian and milk products and egg

Clinical manifestations:

1. Pallor – yes / no
2. Jaundice – yes / no
3. Hyperpigmentation – yes / no
4. Glossitis – yes / no
5. Sub acute combined degeneration* – yes / no
6. Peripheral neuropathy – yes / no
7. Neuro psychiatric symptoms – yes / no

(General Health Questionnaire)

Comorbidities:

1. Diabetes – yes / no
2. Hypertension – yes / no
3. Ischemic heart disease – yes / no
4. Cerebrovascular disease – yes / no

Lab parameters:

1. B12 –
2. Folate –
3. MCV –
4. Haemoglobin –
5. Blood picture – macrocytosis present / absent
6. Pernicious anaemia antibody screen (any of the two antibodies) – positive / negative
7. Homocysteine – high / normal / low
8. Upper GI scopy -

Type of water used for drinking (for more than 50% of the time in the last one year):

Mineral water / aqaguard water / boiled water / chlorinated water / tap water / filter water / well water

Use of betel leaves (at least one per day for the last one year): yes / no

Use of jarda (at least once a day for the last one year): yes / no

Do you know of any extra food supplementation that you may require for being a vegetarian – yes / no

Do you know of this condition called Vitamin B12 deficiency – yes / no

Do you know that you are prone to developing Vitamin B12 deficiency – yes / no

Use of the following drugs (at least once a week for the last one year)

1. Aspirin – yes / no
2. Proton pump inhibitors – yes / no
3. H2 receptor blockers – yes / no
4. Metformin – yes / no

*As evidenced by signs of both the pyramidal tracts and the posterior columns

Annexure 3

Hospital Anxiety and Depression Scale (HADS)

Patients are asked to choose one response from the four given for each interview. They should give an immediate response and be dissuaded from thinking too long about their answers. The questions relating to anxiety are marked "A", and to depression "D". The score for each answer is given in the right column. Instruct the patient to answer how it currently describes their feelings.

A	**I feel tense or 'wound up':**	
	Most of the time	3
	A lot of the time	2
	From time to time, occasionally	1
	Not at all	0

D	**I still enjoy the things I used to enjoy:**	
	Definitely as much	0
	Not quite so much	1
	Only a little	2
	Hardly at all	3

A	**I get a sort of frightened feeling as if something awful is about to happen:**	
	Very definitely and quite badly	3
	Yes, but not too badly	2
	A little, but it doesn't worry me	1
	Not at all	0

D	**I can laugh and see the funny side of things:**	
	As much as I always could	0
	Not quite so much now	1
	Definitely not so much now	2
	Not at all	3

A	**Worrying thoughts go through my mind:**	
	A great deal of the time	3
	A lot of the time	2
	From time to time, but not too often	1
	Only occasionally	0

A	**I can sit at ease and feel relaxed:**	
	Definitely	0
	Usually	1
	Not Often	2
	Not at all	3

D	**I feel as if I am slowed down:**	
	Nearly all the time	3
	Very often	2
	Sometimes	1
	Not at all	0

A	**I get a sort of frightened feeling like 'butterflies' in the stomach:**	
	Not at all	0
	Occasionally	1
	Quite Often	2
	Very Often	3

D	**I have lost interest in my appearance:**	
	Definitely	3
	I don't take as much care as I should	2
	I may not take quite as much care	1
	I take just as much care as ever	0

A	**I feel restless as I have to be on the move:**	
	Very much indeed	3
	Quite a lot	2
	Not very much	1
	Not at all	0

D	**I look forward with enjoyment to things:**	
	As much as I ever did	0
	Rather less than I used to	1
	Definitely less than I used to	2
	Hardly at all	3

A	**I get sudden feelings of panic:**	
	Very often indeed	3
	Quite often	2
	Not very often	1
	Not at all	0

D	**I can enjoy a good book or radio or TV program:**	
	Often	0
	Sometimes	1
	Not often	2
	Very seldom	3

	Scoring (add the As = Anxiety. Add the Ds = Depression). The norms below will give you an idea of the level of Anxiety and Depression.	
	0-7 = Normal	
	8-10 = Borderline abnormal	
	11-21 = Abnormal	

Reference: *Zigmond and Snaith (1983)*

Annexure 4

GENERAL HEALTH QUESTIONNAIRE (GHQ) - 12

General Health Questionnaire

Have you recently:

1. Been able to concentrate on what you're doing?

Better than usual | Same as usual | Less than usual | Much less than usual

2. Lost much sleep over worry?

Not at all | No more than usual | Rather more than usual | Much more than usual

3. Felt you were playing a useful part in things?

More so than usual | Same as usual | Less useful than usual | Much less than usual

4. Felt capable of making decisions about things?

More so than usual | Same as usual | Less useful than usual | Much less than usual

5. Felt constantly under strain?

Not at all | No more than usual | Rather more than usual | Much more than usual

6. Felt you couldn't overcome your difficulties?

Not at all | No more than usual | Rather more than usual | Much more than usual

7. Been able to enjoy your normal day-to-day activities?

More so than usual | Same as usual | Less useful than usual | Much less than usual

8. Been able to face up to your problems?

More so than usual | Same as usual | Less useful than usual | Much less than usual

9. Been feeling unhappy and depressed?

Not at all | No more than usual | Rather more than usual | Much more than usual

10. Been losing confidence in yourself?

Not at all | No more than usual | Rather more than usual | Much more than usual

11. Been thinking of yourself as a worthless person?

Not at all | No more than usual | Rather more than usual | Much more than usual

12. Been feeling reasonably happy, all things considered?

More so than usual	Same as usual	Less useful than usual	Much less than usual

Scoring (General Health Questionnaire -12) – Likert Scale 0, 1, 2, 3 from left to right.
12 items, 0 to 3 each item
Score range 0 to 36.
Scores vary by study population. Scores about 11-12 typical.
Score >15 evidence of distress, Score >20 suggests severe problems and psychological distress